Oral Yeasts Infection Among Patients with Diabetes Mellitus

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Declaration

I declare that the work presented herein was conducted by myself.

Edinburgh, July 1992
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I dedicate this work to my parents

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Abstract

Immunocompromised individuals are prone to opportunistic infections including superficial candidiasis. Individuals with severely suppressed immune functions (AIDS, neoplastic malignancies) as well as pregnant women and diabetics are examples of immunocompromised hosts who have been reported to have a higher prevalence of yeast infections. In this study, diabetic individuals were selected as a model of an immunocompromised group to examine host-pathogen interactions which might be important in colonization and development of disease due to yeasts.

Oral candidiasis is associated with the genetic characteristic of non-secretion of ABH blood group antigens. The general objectives of this project were to assess by epidemiological studies the contribution of non-secretion and environmental factors to colonization by yeasts and development of oral candidiasis among diabetics; and, to assess by laboratory studies effects of the differential distribution of H and Lewis blood group antigens on epithelial cells of secretors and non-secretors on attachment of *Candida albicans*.

Since insulin-dependent diabetes mellitus (IDDM) is distinct from non-insulin dependent diabetes mellitus (NIDDM) with respect to aetiology, pathogenesis and management, these two groups were considered separately. A total of 439 diabetic individuals (231 with IDDM, 205 with NIDDM, 3 with unclassified diabetes) were sampled for carriage of yeasts and oral diseases (denture stomatitis in particular). Yeasts were isolated by swabbing five different sites to assess colonization of different parts of the oral cavity and an oral rinse technique for quantitative assessment of oral carriage.

Yeast isolates were identified by conventional means as well as by the API 20C Auxanogram kit. Results obtained solely with the kit were not always accurate; the most frequently misidentified species was *C. albicans*. The germtube production test in horse serum is, therefore, necessary for accurate identification of this species.

The epidemiological studies emphasized the need to assess results by multivariate analyses. Using Wilk's stepwise multivariate analysis, quantitative carriage, assessed by the oral rinse technique, among individuals with NIDDM who did not wear dentures was associated with glycaemic control (glycosuria $p < 0.01$, plasma glucose $p < 0.05$)); and, non-secretion was a marginally significantly factor ($p = 0.05$). Palatal carriage was, however, associated only with glycaemic control (glycosuria $p <$

0.01). Age was a significant factor associated with carriage of yeasts for individuals with IDDM. When assessed by the palatal swab and oral rinse techniques younger patients were more prone to carriage.

Introduction of a denture altered predisposing factors involved in colonization. For individuals with IDDM, glycaemic control was a significant factor associated with palatal carriage ($p < 0.05$); quantitative carriage assessed by the oral rinse technique was influenced by the presence of retinopathy ($p < 0.05$). Among subjects with NIDDM, palatal carriage of yeasts was associated with continuous wearing of the denture ($p < 0.01$); but, carriage assessed by the oral rinse technique was influenced by plasma glucose levels ($p < 0.05$).

Denture stomatitis was associated with glycaemic control among subjects with IDDM. Patients with NIDDM were prone to develop denture stomatitis if they wore their denture continuously, harboured large numbers of yeasts and were non-secretors of blood group antigens. These results show that predisposing factors involved in the colonization by yeasts and development of denture stomatitis are distinct for individuals with IDDM compared with those with NIDDM.

Host-pathogen interactions underlying the increased susceptibility of non-secretors among non-diabetic individuals and those with NIDDM to colonization by yeasts were explored. Since adhesive ability of microorganisms is associated with colonization, buccal epithelial cells (BEC) from healthy secretor and non-secretor donors were assessed by flow cytometry for their ability to bind yeasts. Two strains of *C. albicans* were examined in these assays. *C. albicans* 2346 expresses an adhesin that binds fucose, the immunodominant sugar of the H and Lewis antigens. *C. albicans* 2023 expresses an adhesin that binds N-acetyl D-glucosamine. There was a trend for BEC from non-secretors to bind more *C. albicans* 2346 compared with BEC from secretors. *C. albicans* 2023 did not show any preferential binding to cells from secretors or non-secretors. Secretors and non-secretors express similar quantities of H Type 2 on their epithelial cells. The increased binding of *C. albicans* 2346 to non-secretors' BEC could not be explained by the presence of a fucose-recognizing adhesin which bound to H Type 1 or Le^b determinants as these are present only on cells from secretors. These results suggested that the $Lewis^a$ antigen present at higher levels on cells from non-secretors might be one of the receptors for *C. albicans* 2346.

The role of Le^a as a receptor for the fucose-recognizing adhesin on *C. albicans*

2346 was examined. Binding of the extracellular polymeric material (EP) to blood group determinants was investigated by enzyme-linked immunosorbent assays (ELISA). Results of the ELISA indicate that all blood group glycoconjugates including the Type 1 precursor bound to EP; but, Lewis^a was bound in greater amounts than the other glycocojugates examined. This suggests that there are in the EP, in addition to adhesins that bind to fucose, adhesins that bind to part of the Type 1 precursor chain. Further investigations need to be carried out to assess the proposed role of Lewis^a antigen as one of the receptors for *Candida* species.

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Chapter 1
Introduction

In order to provide the necessary background to the aims of the study, this chapter will review oral candidiasis and secretion of blood group antigens. The historical aspect, types of superficial disease, epidemiology and pathogenesis of oral candidiasis will be outlined and followed by a review of blood groups and secretor status. The aims of the study will be stated at the end of the introduction.

Candidiasis

1.1 Early Descriptions and Identification of *Candida* as a Causative Agent of Disease

The historical background of *Candida* and candidiasis has been reviewed by Odds (1988) and is summarized here in brief. Candidiasis refers to disease caused by *Candida* species. The term candidosis conveys the same meaning and is used sometimes in preference. Thrush, a form of oral candidiasis, was first reported by Hippocrates in the 4th Century B.C. when he described two cases of oral "aphthae" associated with severe underlying disease.

The organism causing thrush was discovered by Langenbeck (1839) who described a fungus in buccal aphthae of a patient with typhus. Although Langenbeck wrongly suspected that the fungus was the cause of typhus, his description was, nevertheless, one of the earliest to associate a microorganism with a pathological process. Berg (1846) is credited with describing the relationship between the thrush fungus and mouth lesions.

Before 1923, the taxonomy of the thrush fungus was controversial. Gruby (1842) placed Langenbeck's fungus in the genus *Sporotrichum* but Robin (1853) named it *Oidium albicans*. Plaut (1887) isolated a yeast-like fungus from rotting wood which produced lesions resembling oral thrush in chickens. He considered his isolate to be identical with *Monilia candida*, a dimorphic species with a complex taxonomic history. Zopf (1890) named the thrush fungus *Monilia albicans* and gave rise to the name moniliasis for infections due to the thrush fungus.

Since Monilia described the genus of the original botanical molds, Berkhout (1923) proposed the new generic name, *Candida*. *Candida* derived from Latin for "white robe worn by candidates for the Senate" and *albicans* is the Latin participle for *albicare*, "to whiten".

Many yeasts (88) have been isolated from human sources. Most of the species are rare; the eight most frequently encountered human isolates are *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata*, *Candida guillermondii*, *Candida krusei*, *Candida kefyr* and *Candida viswanithii* (reviewed by Odds, 1988).

Candida infections of virtually every tissue in the human body have been reported. Their systematic classification creates problems. The general terms of superficial and deep candidiasis are used here. Superficial includes those infections that are visible (candidiasis of oral and aural cavities, genitalia, skin and nail); and, deep or systemic candidiasis refers to all other sites of infection. This classification is useful in that it separates the common (superficial) and more easily diagnosed forms from the deep and comparatively difficult to diagnose forms.

One of the most common forms of candidiasis is oral infection associated with dentures, denture stomatitis. The various forms of oral candidiasis have been reviewed by Soames and Southam (1985). They are considered below with respect to prevalence, clinical and histological appearances, aetiology, pathogenesis, and treatment.

1.2 Oral Candidiasis

1.2.1 Pseudomembranous candidiasis

This disease is most prevalent in the extremes of age and among the terminally ill. It also occurs with more serious underlying conditions: diabetes; leukaemia and other malignancies; and, the acquired immunodeficiency syndrome (AIDS). A prevalence of 10% and 15% has been recorded for debilitated elderly patients and a lower incidence (7%) has been reported among neonates (Odds, 1988).

Clinically it presents as a thick white coating on the mucosa which can be wiped away to leave a red, raw and often bleeding base. Histologically the affected area shows a hyperplastic epithelium with the superficial layers infiltrated by both hyphae

and spores and inflammatory cells which are predominantly neutrophils. The neutrophils can accumulate to form microabscesses. The superficial epithelial cells are separated by inflammatory oedema from the deeper layer; and, it is along this plane that the pseudomembrane separates when the lesion is firmly wiped. In addition, there is infiltration of the lamina propria by acute and chronic inflammatory cells.

C. albicans is the common cause of oral thrush although occasionally reports implicate other species. The maternal birth canal is a major source of neonatal infections but teats, toothbrushes and other fomites have been implicated (Kozinn *et al.*, 1958).

Newborns are probably susceptible to candidal infection because of their immature antimicrobial defences. In paired maternal/umbilical blood samples higher titres of anti-*Candida* antibodies and lymphocyte transformation responses to *C. albicans* were found in the maternal blood than in neonatal blood (Schneider *et al.*, 1986). Antibodies in cord blood contain mainly IgG whereas maternal blood often has IgA and IgM antibodies to *Candida* as well (Mathus *et al.*,1979).

Cox (1986) found that at birth adherence of *C. albicans* to BEC was half that measured 4 days later. This 4 day interval of relative protection from adherence of *C. albicans* is of the same order as the usual delay before onset of oral thrush in neonates. No relation between candidal infection and local trauma, debility, prematurity, low birth weight, breast or bottle feeding has been found (Ludlam, 1942; Kaloyannides, 1968).

Pseudomembranous oral candidosis is often treated with oral antifungal agents. The majority of cases are effectively cured with this treatment (Alban, 1970; Cohen *et al.*, 1974; Botter, 1980).

1.2.2 Acute atrophic candidiasis

This form of candidiasis is usually seen in patients undergoing prolonged antibiotic or steroid therapy. The common name is antibiotic sore tongue because the tongue is most frequently affected.

Clinically, the lesion is red and often painful and resembles thrush without the overlying pseudomembrane. Histologically the epithelium is atrophic with only

occasional yeast hyphae and spores penetrating the superficial layers of non-keratinized epithelium. There is an associated inflammatory infiltrate in both the epithelium and in the underlying connective tissue.

The aetiology of this condition is not known. It is thought that the antibacterial therapy alters the oral bacterial flora and allows organisms such as *Candida* to flourish. Concomitant administration of antifungal agents and change or termination of antibiotic and/or steroid therapy is usually the treatment of choice in this condition.

1.2.3 Chronic hyperplastic candidiasis (candidal leukoplakia)

The prevalence of leukoplakia (0.2-4%) varies in different parts of the world mainly because of different diagnostic criteria. The prevalence of candidal leukoplakia is generally less than that of leukoplakia; invasion of the lesion by yeasts has been documented in biopsies from 6-90% of cases (Jepson *et al.*,1965; Renstrup, 1970; Daftary *et al.*, 1972; Eversole *et al.*, 1986)

Clinically this condition presents as a persistent white patch on the oral mucosa which is indistinguishable from leukoplakia. The dense, opaque white plaques can be detached by firm scraping; and, identification of hyphae assists in diagnosis. A number of these lesions have areas of erythematous mucosa within the outline of the plaque producing a speckled appearance. They usually occur on the inside surface of one or both cheeks. Among homosexual men the tongue is involved more often than the cheeks (Eversole *et al.*, 1986). Males are affected more frequently than females (Arendorf *et al.*, 1983).

Histological appearance is that of parakeratosis and acanthosis. Many of the cells in the parakeratinised surface are separated by oedema and numerous polymorphs which often collect to form microabscesses. *Candida* hyphae invade the parakeratin more or less at right angles to the surface but never penetrate deeper. A number of acute and chronic inflammatory cells are present throughout the prickle cell layer; and, there is a mixed chronic inflammatory cell infiltrate in which plasma cells are often prominent in the subepithelial connective tissue. The absence of the infected parakeratin layer might account for the erythematous appearance seen clinically.

It has not been conclusively shown whether these lesions are primarily leukoplakia with secondary candidal infection or whether they are primarily chronic candidal infection which in time lead to epithelial hyperplasia. Krogh *et al.* (1986)

have suggested that there might be phenotypic differences between *C. albicans* strains associated with leukoplakia and with normal epithelium in the same patient.

The lesion is associated with the following: smoking; denture wearing; iron and folate deficiencies; raised antibody titre to *C. albicans*; defective cell mediated immunity (Jenkins *et al.*, 1977; Mackie *et al.*, 1978; Arendorf *et al.*, 1983). Langerhans cells show a more irregular distribution in candidal leukoplakia lesions than in normal epithelia (Daniels *et al.*, 1985). The speckled type of leukoplakia is more often associated with *Candida* invasion and malignant changes; but, these should not be taken as evidence for a causal association between *Candida* and atypia (Jepsen *et al.*, 1965; Daftary *et al.*, 1972).

Candidal leukoplakia sometimes responds to prolonged topical antifungal therapy, but surgical resection is necessary in some cases. In asymptomatic leukoplakia, treatment is instituted because both erosive and non-erosive forms have been reported to undergo malignant transformation (Cawson and Lehner, 1968; Mackie *et al.*, 1978; Daftary *et al.*, 1972).

1.2.4 Chronic mucocutaneous candidiasis (CMC)

This is a rare group of disorders associated with extensive and chronic candida infection of mucosae, nails and skin; the oral mucosal involvement is usually a dominant feature. In 90% of patients with CMC, chronic pseudomembraneous oral candidiasis is the earliest lesion to appear, and it frequently precedes allied conditions such as endocrinopathies. Persistent onychia and paronychia are nearly as common as oral lesions. Skin lesions when present appear principally on the face, ears, neck and shoulders. Disseminated candidiasis is hardly ever seen (Odds, 1988).

Chronic mucocutaneous candidiasis is not a single disease entity hence its classification is difficult. Wells (1973) defined four subgroups of CMC patients on the basis of hereditary factors as well as clinical features (Table 1.1). Various modifications of the above classification appear from time to time. Most patients with CMC fall in Group 3 with single or multiple endocrinopathies: hypoparathyroidism, hypoadrenocorticism, hypothyroidism and/or diabetes mellitus.

Table 1.1 Subgroups of Chronic Mucocutaneous Candidiasis

Group 1:	Familial CMC with autosomal recessive inheritance
Group 2:	CMC with unknown/obscure familial factors
Group 3:	CMC associated endocrinopathy with or without evidence of autosomal recessive inheritance of the endocrinopathy.
Group 4:	Mature onset CMC.

CMC is most often associated with defects in cell mediated immunity (CMI). Cutaneous delayed-type hypersensitivity (DTH) response to *Candida* antigens is absent in over 80% of patients tested compared with 16%-37% of controls (Palmer *et al.*, 1974; Rothschild *et al.*, 1976). Lymphocyte transformation and production of macrophage inhibition factor in response to *Candida* antigens *in vitro* were impaired and correlated with absent cutaneous responses in many instances (Ferguson *et al.*, 1977; Sohnle *et al.*, 1977). *In vitro* studies suggest that degradation products of *Candida* mannans might mediate the suppressive effects (Podzorski *et al.*, 1990). These deficiencies seem to be the consequence of more fundamental disorders in lymphocyte function, particularly in co-operative interactions between T cell subsets (Kirkpatrick *et al.*, 1971). *Candida* extracts act as specific antigens in these tests and not as mitogens (Ausiello *et al.*, 1986).

Abnormal ratios of T_{helper}:$T_{suppressor}$ have been noted, a situation similar to that seen in AIDS patients (Gupta *et al.*, 1979). Lymphoctye suppressor factor (Fischer *et al.*, 1978; Lee *et al.*, 1986), abnormal immunoglobulin levels (Van Scoy *et al.*, 1975), presence of autoimmune antibodies and immune complexes (Sohnle *et al.*, 1976; Zouali *et al.*, 1983), complement factor (Drew, 1973) and granulocyte defects (Van der Meer, 1978; Bortolussi *et al.*, 1981) have also been noted. The multiple disorders reflect the heterogeneity of CMC and obscure attempts to elucidate the pathogenic mechanisms.

In many cases there is a clear evidence of hereditary linkage in CMC. The HLA B7 haplotype frequency is increased among these patients (Kirkpatrick, 1984). Since CMC appears to be the result of one or more minor defects in host defences, it is unlikely that a single gene can be incriminated (Rothchild *et al.*, 1976).

C. albicans accounts for a majority of cases of these infections. As in other

superficial forms of candidiasis, *C. albicans* does not penetrate appreciably through the epithelium. There is a substantial infiltration of acute and chronic inflammatory cells. Systemic antifungal treatment can usually achieve remission; however, recurrences are common (Hay, 1981).

1.2.5 Chronic atrophic candidiasis (denture stomatitis)

Denture stomatitis is the commonest form of disease associated with *Candida.* It is found in 24-60% of denture wearers (Nyquist, 1952; Budtz-Jorgensen *et al.*, 1975). Clinically, it presents as chronic erythema and oedema of the portion of the palate that comes into contact with the denture; lower denture bearing areas are hardly ever involved.

The histological appearance is of parakeratosis of the palatal epithelium with vacuolation and neutrophil infiltration of the upper spinous layer. There is lymphocyte infiltration of deeper spinous layers and diffuse subepithelial inflammation without any evidence of fungal penetration.

Denture stomatitis is an inflammatory reaction to yeasts promoted by occlusion of the palatal mucosa with a denture. The inflammation appears to be a response to the growth of large numbers of yeasts in the space between the denture and the palatal mucosa; yeasts do not invade the mucosa. Availability of sucrose and metabolic acid production by yeasts are thought to contribute to the pathology (Olsen and Birkland, 1976; Samaranayake *et al.*, 1982). Folate deficiency has been associated with denture stomatitis (Jenkins *et al.*, 1977; Samaranayake, 1986); but, iron deficiency is not a factor (Jenkins *et al.*, 1977). Yeast virulence factors such as proteinase production might act as inflammatory aggressins; however, there was no correlation between proteolytic activity of yeasts isolated and the severity of the inflammation (Budtz-Jorgensen, 1970).

No differences in CMI, leucocyte counts or candidicidal properties are apparent between patients and controls (Davenport and Wilton, 1971; Budtz-Jorgensen, 1973; Bergendal *et al.*, 1977; Taafe *et al.*, 1985). Serum antibody levels against *C. albicans* are elevated but do not correlate with severity of infection (Budtz-Jorgensen, 1972; Bergendal and Holmberg, 1982). Treatment is usually aimed at reducing the yeast concentration; removal of the denture together with antifungal treatment is most successful (Carter *et al.*, 1986).

1.3 Epidemiology of Oral Carriage of Yeasts and Denture Stomatitis

Varying frequencies for isolates of yeasts from similar sites in nominally similar subjects are reported. Patient selection and sampling methods are possible sources of variation. Oral carriage is best assessed by imprint culture method than from saliva or a mouthwash sample (Arendorf and Walker, 1979; Oliver and Shillitoe, 1984). The sensitivity of an imprint culture was equal to that of a 60 s mouthrinse (Samaranayake *et al.*, 1986). Enrichment culture of swabs increased the isolation of yeasts (Schonheyder *et al.*, 1984).

Identification can further contribute to apparent disparities in carriage rates of yeasts (Odds, 1988). Many studies state frequencies of recoveries of *C. albicans*, but the tests used for identification of this species might not be stated or are based on criteria that are presumptive. In some cases it is likely that "*C. albicans*" is synonymous with "yeast" and vice versa. Dramatic effects on isolation rates of different species were reported within the same laboratory following changes in methods of identification (Hurley and Morris, 1964; Hurley *et al.*, 1973).

Several studies have detailed the relative proportions of different yeast species isolated from man. The general conclusion is that *C. albicans* accounts for 60-80% of oral isolates. *C. glabrata* and *C. tropicalis* are both found with modest frequencies (~7%) (Odds, 1988). *S. cerevisiae* was the predominant species found in the mouths of patients in Zaire (Melbye *et al.*, 1985). It is not known whether the difference is a true regional variation of yeast flora (perhaps due to diet) or approaches to species identification.

The prevalence of denture stomatitis varies among populations sampled; but, 24-60% of denture wearers have been reported to be affected. This might be due to differences in susceptibility of the sample populations. *C. albicans* is most frequently isolated from the lesion. Although denture stomatitis is caused by yeasts (Cawson, 1963; Budtz-Jorgensen, 1974), harbouring yeasts does not inevitably lead to the development of the disease. In the study to elucidate mechanisms underlying the diseased state, factors which influence carriage are as important as those which influence the development of denture stomatitis. These can be broadly classified as genetic and environmental. The latter consists of four categories: (a) digression from physiological status (diabetes, malignancy); (b) age; (c) physical factors including presence of a prosthesis, diet, smoking; and (d) iatrogenic factors (antibiotics,

corticosteroids).

1.3.1 Genetic factors

1.3.1.1 Gender

Only one study reported that carriage of *C. albicans* was more frequent among female diabetic individuals compared with males (Amato and Pecora, 1983). There have been reports of females being more susceptible to denture stomatitis than males (Cawson 1966; Davenport, 1970; Challacombe, 1986); but, other workers found no sex bias (Budtz-Jorgensen *et al.*, 1975; Lamey *et al.*, 1988). This discrepancy might be due to females wearing dentures more frequently and for longer periods than males; and, therefore being over-represented in the sample population.

1.3.1.2 Blood group and secretor status

Carriage of *C. albicans* in healthy individuals was associated with blood group O and non-secretion of blood group antigens; these associations were independent of each other (Burford-Mason *et al.*, 1988). Among individuals with non-insulin dependent diabetes (NIDDM), blood group O non-secretors were more frequently colonized than O secretors. Individuals with insulin-dependent diabetes (IDDM) did not show this relationship (Blackwell *et al.*, 1989a). Another study which did not differentiate the blood groups of their subjects similarly found individuals with NIDDM who were non-secretors prone to oral carriage of yeasts (Darwazeh *et al.*, 1990). The latter study is in contrast to a report by the same research group which found no significant association between secretor status and carriage of yeasts or development of denture stomatitis among subjects with diabetes (Lamey *et al.*, 1988).

Non-secretion of ABH antigens has been associated with oral candidiasis; and, non-secretors are over-represented among pregnant women with vaginal candidiasis (Thom *et al.*, 1989). The mechanisms by which secretor status might affect carriage or disease are discussed in Section 1.7.2.

1.3.2 Digression from physiological status

1.3.2.1 Diabetes

Several investigators have reported higher frequencies of oral yeast carriage among individuals with diabetes mellitus (Weinstein *et al.*, 1959; Johnston *et al.*, 1967; Barlow and Chattaway, 1969; Cambon *et al.*, 1979; Lamey *et al.*, 1988). Not all investigators found higher yeast prevalences in the mouths of diabetics (Young *et al.*, 1951; Mehnert and Mehnert, 1958; Loiselle *et al.*, 1964; Peters *et al.*, 1966; Tapper-Jones *et al.*, 1981; Thorstensson *et al.*, 1989). Some authors have found a higher concentration of yeasts in the mouths of diabetic individuals (Peters *et al.*, 1966; Tapper Jones *et al.*, 1981).

In studies where individuals with diabetes were compared with non-diabetic subjects, the prevalence of denture stomatitis was higher in the former group (Lamey *et al.*, 1988); however, Phelan and Levin (1986) found no difference in the prevalence of denture stomatitis between the American Indians with diagnosed diabetes and/or elevated plasma glucose levels compared with non-diabetic individuals. This discrepancy might be due to the difference in the populations examined; most American Indians (> 95%) are secretors of blood group antigens and NIDDM is the commonest form of diabetes in this population (Mourant *et al.*, 1976; Blackwell, 1989).

The mechanisms by which diabetes increases host susceptibility to oral carriage and disease are not clear. Physical factors such as salivary flow, pH and buffer capacity have been compared between diabetic and non-diabetic individuals. The results are controversial because of vague and differing classification of diabetes in different studies (Kjellman, 1970; Faulconbridge *et al.*, 1981; Sharon *et al.*, 1985; Tenovuo *et al.*, 1986; Banoczy *et al.*, 1987). In a well defined study of individuals with IDDM, there was no difference in salivary pH or buffer capacity between well controlled patients with long term or short term IDDM compared with controls. There was a small significant difference of mean salivary flow levels between diabetics and controls. The physiological significance of this difference is probably negligible (Thorstensson *et al.*, 1989).

Salivary glucose levels are slightly but significantly increased among individuals with IDDM compared with non-diabetics (Thorstensson *et al.*, 1989). It has been

proposed that high blood and tissue glucose levels favour growth of *Candida* (Knight and Fletcher, 1971; Odds *et al.*, 1978). In a study by Rayfield *et al.* (1982), a striking overall correlation between prevalence of disease and mean fasting plasma glucose levels was found; they concluded that glycaemic control was important in reducing susceptibility to infection.

Defective chemotaxis, adherence, phagocytosis and killing of bacteria by granulocytes from diabetic individuals have been reported by some and refuted by other workers. These differences might be due to different assay techniques and time of sampling phagocytic activity (Davidson *et al.*, 1984). Reports of phagocytosis of *Candida* species are similarly controversial. Wilson and Reeves (1986) noted no difference in phagocytosis of *C. albicans* by neutrophils from diabetic subjects; however, neutrophils isolated from diabetic subjects showed impaired phagocytosis of *C. guillermondii* (Davidson *et al.*, 1984), and monocytes had a decreased ability to phagocytose *C. albicans* (Geisler *et al.*, 1982). The decrease in phagocytic function is thought to result from a reaction of glucose with proteins concerned with phagocytosis resulting in the blocking of serum opsonins (C3b, Fab) and granulocyte receptors (CR3, FCR) (Davidson *et al.*, 1984).

Wilson and Reeves (1986) measured a decrease in the ability of neutrophils from individuals with diabetes to kill *Candida* when the assay was done in the presence of 50 mM glucose or 20 mM β-hydroxybutyrate alone or in combination. They suggested that in diabetics, conversion of glucose to sorbitol reduces the available concentration of NADPH; and, this inhibits the oxidative killing mechanisms of the neutrophils (Wilson *et al.*, 1987). It is interesting that oxidative activity following engulfment of *C. albicans* by neutrophils is decreased compared with non-diabetic controls and was independent of plasma glucose levels (Qvist and Larkin, 1981).

With respect to specific immunity, there is no difference in lymphoproliferative responses to *C. albicans* by lymphocytes from diabetics compared with controls (Ragab *et al.*, 1972). No information is available about the mucosal immunity against *Candida* in diabetic individuals.

1.3.2.2 Malignant diseases

The overall prevalence of yeasts in the mouths of normal subjects is generally of a lower order than in those who have sought medical attention. *Candida* carriage is very common among cancer patients, especially those with leukaemia; and, it is

enhanced by chemotherapy and radiotherapy. Systemic as well as oral candidiasis are more prevalent particularly during phases of neutropenia; the prevalence of denture stomatitis is unknown (Odds, 1988).

1.3.2.3 Age

Carriage of yeasts in the mouths of neonates is an exception rather than the rule; but, within the first year of life, most infants appear to acquire oral yeast flora. Most cases of oral infection within the first few days of life arise primarily because of maternal contamination of babies with yeasts in the birth canal (Kozinn *et al.*, 1958; Tarr, 1980). The relative vulnerability of the very young stems from the immaturity of their antimicrobial defences.

The prevalence of yeast in the adult mouth has been shown to rise with age. The effects of age per se are not always easily separated from diseases and medical treatment associated with increased age (Marples, 1960; Smits *et al.*, 1966). Denture stomatitis has not been reported to be directly related to age.

1.3.3 Physical factors

1.3.3.1 Diet

Oral yeast concentration in monkeys rose in association with high sugar diet (Bowen, 1974). Initiation and degree of inflammation of denture stomatitis in man is increased by repeated sucrose rinses (Olsen and Birkeland, 1976). It has been suggested that diets rich in refined carbohydrates can cause disease by a process of enhancing adhesion of yeasts which promotes colonization. The availability of nutrients with the associated production of metabolic products such as pyruvates and acetates might damage the mucosa directly, provide an acid millieu for yeast proteinase activation and lead to the development of denture stomatitis (Samaranayake and MacFarlane, 1985).

1.3.3.2 Prosthesis

Several workers have detected a higher prevalence of oral yeasts among denture wearers compared with individuals who did not wear dentures (Peters *et al.*, 1966;

Budtz-Jorgensen *et al.*, 1975; Berdicevsky *et al.*, 1980; Tapper-Jones *et al.*, 1981; Mitchell, 1982; Vandenbussehe and Swinne, 1984; Fisher *et al.*, 1987; Lamey *et al.*, 1988; Hill *et al.*, 1989). It is possible that the main effect of the denture is to increase yeast concentration rather than to establish yeast populations in mouths that did not previously carry them. Similar considerations apply to removable and fixed orthodontic appliances (Addy *et al.*, 1982; Arendorf *et al.*, 1983). In babies, dummies might serve as occlusive foreign bodies leading to elevated oral *Candida* concentrations. High yeast levels are found in patients who wear dentures continuously (Budtz-Jorgensen *et al.*, 1983).

Continuous wearing of dentures has been found to influence the development of denture stomatitis (Ritchie *et al.*, 1969; Arendorf and Walker, 1979; Tapper-Jones *et al.*, 1981). This is hardly surprising considering that denture stomatitis is induced only in the presence of a denture and that removal of the denture causes remission even in the absence of treatment with antifungal agents. Trauma due to poor fit and/or occlusion of the denture has been found to influence the development of the lesion (Nyquist, 1952; Ritchie *et al.*, 1969; Budtz-Jorgensen and Bertam, 1970).

1.3.3.3 Smoking

Significant increases in oral *Candida* carriage among smokers have been reported in both diabetic and non-diabetic sample populations (Tapper-Jones *et al.*, 1981; Arendorf *et al.*, 1983). Other studies did not find this association (Bastiaan and Reade, 1982; Hill *et al.*, 1989). The relationship between smoking and denture stomatitis has not been explored. Smoking has been reported to be a very significant factor in the development of candidal leukoplakia (Arendorf *et al.*, 1983).

1.3.4 Iatrogenic factors

Evaluation of iatrogenic factors is not easy. It is often difficult to differentiate the contributions made by the underlying illness from the treatment.

In theory, most antibacterial agents can disrupt microbial populations that normally compete with yeasts for nutrients; therefore, yeasts are able to multiply more readily. In practice, the extent of the "antibiotic effect" is difficult to assess. It is often considered that broad spectrum antibiotics are more likely to lead to *Candida* overgrowth than drugs with a more limited range of targets. Tetracycline increased

the prevalence of oral *Candida* in a longitudinal study; but, in a cross-sectional study in which tetracycline-treated and untreated patients were of comparable clinical status, there was no difference in oral *Candida* carriage (McKendrick *et al.*, 1967).

Elimination of bacterial competition is almost certainly the mechanism by which antibiotics affect *Candida* numbers *in vivo*. Certain antibiotics might have immunosuppresive properties that reduce host resistance to *Candida*. Erythromycin, cotrimoxazole and several aminoglycosides reduced neutrophil candidicidal activity *in vitro*. Penicillin, tetracycline, chloramphenicol, gentamicin, azlocillin and carbenicillin do not show this effect (Bridges *et al.*, 1980; Ferrari *et al.*, 1980; Hawkey *et al.*, 1983).

Corticosteroids are compounds with pronounced anti-inflammatory and immunosuppressive effects. Surveys have shown a higher prevalence of yeasts in patients receiving corticosteroid therapy (Johnston *et al.*, 1967; Shastry et al., 1969). Immunosuppressive effects might not be the only means by which corticosteroids can influence candidiasis. There is an intracellular steroid binding protein in *C. albicans* with a high specificity for cortisone and progesterone (Loose *et al.*, 1981). The function of this protein and the effect of corticosteroid on yeast growth are not known as yet (Loose and Feldman, 1982; Powell and Drutz, 1983). Some authors have claimed that steroid inhalers result in increased oral *Candida* carriage, but other workers did not find this effect (Kerrebijn, 1976; Pringleton *et al.*, 1977; Toogood *et al*, 1984).

The effect of antibiotics and corticosteroids on denture stomatitis have not been studied in man. Vogt (1979) concluded that the effect of corticosteroid inhalers in the development of superficial candidiasis was minimal. The role of antibiotics in superficial candidiasis is also found to be minimal in two large studies of hospitalized patients (Caldwell and Cluff, 1974; Walker *et al.*, 1979).

1.4 Pathogenesis Of Candidiasis

Candida species are opportunistic pathogens which can be regarded as possessing multiple properties, each with a low propensity for enhancing infectivity but none necessarily dominant. Even in combination, these are unlikely to overcome fully intact host defences. This section outlines the potential virulence factors of *Candida* and summarizes the results of several studies of host defense against *Candida*.

1.4.1 Virulence determinants of *Candida*

1.4.1.1 Adherence to host surfaces

The ability of yeasts to adhere to host cells corresponds to their relative virulence. Among *Candida* species, *C. albicans* generally adheres to epithelial and other mammalian surfaces better than others such as *C. tropicalis* and *C. parapsilosis* (King *et al.*, 1980; Critchley and Douglas, 1985).

For *C. albicans*, there is general agreement that hyphal germ tubes adhere to host surfaces better than yeast blastospores (Kimura and Pearsal, 1978; King *et al.*, 1980). There is variation in the adherence of yeasts to different host cells. Yeasts were more adherent to epithelial cells from diabetic and pregnant women than those from other women (Lehrer *et al.*, 1983). In neonates, buccal epithelial cell (BEC) are less able to bind *C. albicans* at the time of birth than just a few days later. Epithelia from infants colonized with *C. albicans* bind more yeasts than epithelia from non-carriers (Cox, 1986). *C. albicans* strains isolated from invasive *Candida* infections adhere better than those from carriers (Douglas *et al.*, 1981; McCourtie and Douglas, 1984). Adherence capabilities clearly play a major part in the pathogenesis of candidiasis.

1.4.1.2 Phenotypic variability

The ability to express different phenotypic properties is an advantage which might account for the ability of *C. albicans* to infect a wide range of host environments (Soll *et al.*, 1987). The only evidence linking phenotypic variation *in vitro* with virulence *in vivo* is the switching of colony phenotype occurring with each new vaginal candidal infection. Restriction fragment length polymorphism confirmed that the multiple phenotypes represented the same *C. albicans* strain (Soll *et al.*, 1989). Another example of phenotypic variation is the observation that sub-populations of *C. albicans* adhered more avidly to acrylic surface *in vitro* (McCourtie and Douglas, 1985).

1.4.1.3 Toxins

Suggestions that *C. albicans* might contain toxins analogous to bacterial endotoxins have been explored. Formalin killed blastospores of several *Candida*

species and soluble supernatants from mechanically disrupted *C. albicans* injected intraperitoneally were lethal for mice. Other biological activities claimed for the toxins were pyrogenicity (rabbits), induction of hypotension (dogs), anaphylaxis (rats), histamine release (rats) and complement activation (rodents) (reviewed by Odds, 1988).

Fractions of *C. albicans* prepared by mechanical and chemical methods were tested for toxicity in three animal models. Only the cell wall glycoprotein fraction had toxic properties. It was pyrogenic in rabbits and lethal to mice provided they were pretreated with actinomycin D (Cutler *et al.*, 1972). The effects of such toxins are different from those of bacterial toxins. Their potency is several orders of magnitude less; this is consistent with the status of *C. albicans* as an opportunistic pathogen that contains some molecules with a low but detectable biological potency.

1.4.1.4 Enzymes

Theoretically, hydrolytic enzyme activities expressed on the surface of microbial pathogens could cause damage to host cells *in vivo*. For *C. albicans*, there are two main candidates, a proteinase and a phospholipase; but, incontrovertible evidence for a pathological role is lacking for both.

C. albicans proteinase has been shown by immunofluorescence to be synthesized in infected tissues *in vivo*; and, antibodies to the purified enzyme are found predominantly in patients with visceral *Candida* infections (MacDonald and Odds, 1980; Ruchel, 1981). The non-invasive nature of proteinase-deficient strains has been documented (MacDonald and Odds, 1983). Secretion of proteinase *in vitro* is a ubiquitous property of *C. albicans* isolates, common in *C. tropicalis*, occasional in *C. parapsilosis* and very rare or absent in other species. The distribution of its activity coincides with the rank order of virulence of *Candida* species (Odds, 1985).

Against the view that *C. albicans* proteinase is a virulence attribute is the inconsistent correlation between gross proteinase production in plate tests and strain virulence (Schorlemmer *et al.*, 1977; Germaine and Tellefson, 1981) or severity of inflammation (Budtz-Jorgensen, 1970). The neutral pH of saliva precludes the enzyme's activity in the mouth; however, it has been suggested that yeast metabolites can create an acidic micro-environment conducive to the action of the proteinase (Samaranayake and MacFarlane, 1985).

The role of *C. albicans* proteinase in the pathogenesis of candidiasis is unknown. A proteinase inhibitor, Pepstatin A, reduced mucosal adherence of *C. albicans* (Edison and Manning-Zweerink, 1988). Reports of enzymes with molecular characteristics almost identical to those of the acid proteinase have shown lytic activity against human stratum corneum and collagen (Hattori *et al.*, 1984; Kaminishi *et al.*, 1986). MacDonald and Odds (1983) suggested the enzyme might be involved in fungal resistance to phagocytosis; but, Walter *et al.*, (1986) have shown that proteinase-secreting cells are killed more readily by polymorphs than yeasts that do not secrete the enzyme. They suggest that the enzyme might activate candidacidal proteins.

Phospholipase activity is secreted in growth media by *C. albicans* (Banno *et al.*, 1985). Barrett-Bee *et al.* (1985) found an approximate correlation between phospholipase activity, mouse lethality and adherence of yeast to BEC, the only evidence linking the enzyme with virulence.

1.4.1.5 Dimorphic change in *C. albicans*

Filamentous phase cells are commonly seen in infected tissues; but, yeasts are also present, and no one form seems to predominate. Mutant strains incapable of yeast-mycelial transitions can establish infections (Shepherd, 1985). This, however, does not exclude the possibility that one form might be more efficient at establishing infection than the other. A variant which could not form germ tubes was less virulent than its parent; and, reversion to germ tube formation restored virulence (Sobel *et al.*, 1984). The hyphal tip is most frequently seen in micrographs to penetrate the epithelium (Polack and Grenson, 1973); and, it adheres better to BEC than the yeast form (*vide supra*). It is possible that the ability to interconvert between blastospores and hyphae is an important pathogenic mechanism contributing to evasion of host defences at different stages of the infectious process.

1.4.2 Determinants of host resistance to *Candida* infections

The sophistication of the host defence system compared with the few virulence armaments proposed for *Candida* underlines the fact that *Candida* species can invade superficially only when there is a defect in host response and deeply only when the defects are numerous and serious. Host defenses include both innate and specific mechanisms summarized below.

1.4.2.1 Mechanical barrier

An intact skin surface does not prevent cutaneous *Candida* infection since it is easily overcome in CMC. The skin is mechanically intact, but the patient has defects in cellular immune responses. Also, when the skin is occluded *Candida* species are able to penetrate the epidermis. An intact oral epithelium is present before onset of pseudomembranous candidiasis. There is evidence to suggest that zinc, iron and pyridoxine deficiency might predispose to superficial candida infection because of the poor quality of the epithelium in such cases (Higgs, 1973; Fletcher *et al.*, 1975; Edman *et al.*, 1986).

1.4.2.2 Non-specific antimicrobial substances

Most secretions and fluids of the mammalian host contain substances that act non-specifically to inhibit or kill microbial invaders. For *Candida*, there are reports of inhibitory substances in or on the skin, the conjuctiva, and saliva (Aly *et al.*, 1972). Methodological differences might explain why some studies found saliva inhibitory and others did not. Bartels *et al.* (1967) found that saliva induced zones of inhibition in lawns of *Candida* species on agar containing high concentrations of tetrazolium salts. This method is open to the criticism that the physicochemical nature of the test fluid (pH, ionic strength) might inhibit the fungus rather than a particular inhibitory molecule. Knight and Fletcher (1971) found no evidence of non-specific anti-fungal activity in saliva, apart from the growth impairing effects of bacteria which exhaust the nutrients. In contrast, Pollock *et al.* (1984) have purified histidine-rich polypeptides from saliva and shown them to be potent inhibitors of *C. albicans*.

Lysozyme is only weakly inhibitory for *C. albicans* (Kayama, 1970). Interferons provide no significant protection against intravenous *C. albicans* challenge in mice (Umenai *et al.*, 1978). Non-specific inhibitors of *C. albicans* in serum might protect against intravenous challenge (Hasenclever, 1978).

1.4.2.3 Complement

All yeast mannans activate complement via the alternative pathway (Ray *et al.*, 1979). The role of complement as an opsonin for intracellular killing of *C. albicans* by neutrophils has been suggested. Killing of *C. albicans* is much reduced in sera

from animals congenitally deficient in C3 but not in sera devoid of C2, C5 or C6. Antibodies to C3 reduced the opsonic effect of serum on intracellular killing (Morrison and Cutler, 1981).

Animal studies confirm a requirement for complement activation to ensure elimination of *Candida in vivo*; but, they do not distinguish between complement activation as a leukocyte chemotactic factor from its opsonic activity. Guinea pigs pretreated with cobra venom factor (to deplete components C3 and C5-C9) were significantly more susceptible to intravenous *Candida* challenge (Gelfand *et al.*, 1978). Lyon *et al.* (1986) found that mice deficient in C5 were more severely affected by *C. albicans* challenge than those with intact C5, but they developed identical humoral and cellular immune responses to the yeasts. This suggests that their complement deficiency was a significant factor only at the time of initial microbial challenge and was less important later.

Complement-binding protein receptors (analogues of human CR2 and CR3) have been reported to occur exclusively in hyphal and germtube phases and not in the yeast phase; and, they have been found on *C. albicans* and *S. cerevisiae* only. They have been suggested to be virulence factors. These complement receptors might participate as co-factors in the enzymatic degradation of bound complement, promote clumping of the organism, and compete with phagocyte receptors for the complement fractions thus evading phagocytosis (Edwards *et al.*, 1986; Calderone and Braun, 1991). Infection might be facilitated by the presence of CR3 analogue on *C. albicans*. The human CR3 is an integrin - a member of a gene family involved in interaction between cells. The CR3 analogue of *Candida* has some nucleotide sequence homology with human CR3. Their role in adherence of *C. albicans* to host cells can be postulated since integrin superfamily members mediate binding reactions (reviewed by Cooper, 1991).

1.4.2.4 Phagocytosis and intracellular killing of *Candida*

The ability of neutrophils and tissue macrophages to ingest and kill *Candida* species is a singularly important mechanism enabling the host to remove and destroy yeast cells. Many lines of evidence indicate the importance of phagocytes in host defence against invasive, deep-seated candidiasis: leukopenic patients are more vulnerable to disseminated *Candida* infections than comparable patients with normal leukocyte counts (Fanci *et al.*, 1984). Among patients with AIDS or CMC in whom

lymphocyte-based immunity is impaired but phagocytic function is normal, visceral spread of *Candida* is highly unusual. Candidiasis is restricted to skin and mucous membranes. It has been argued that phagocytic cells are major components of defense against deep-seated candidiasis but are less important in the defence of superficial body sites. This is a simplistic view because granulocytes are part of the immune system's effector mechanisms in eliminating superficial candidal infections.

The relative contributions of neutrophils and macrophages to defence against candidiasis are not easy to determine. From the histological sequence of events in mice following intratracheal challenge of mice with *C. albicans*, resident macrophages were the first line of defence supported by neutrophils (Nugent and Onofrio,1983). For example, carageenan, a substance toxic to mouse macrophages but which also stimulates a neutrophil leucocytosis, does not increase vulnerability of mice to intratracheal challenge and reduces their susceptibility to intravenous *C. albicans* (Hurtrel and Lagrange, 1985; Lal *et al.*, 1986).

Some studies show that yeast cells are more vulnerable to intracellular killing than preformed hyphae (Davies and Denning, 1972; Scherwitz and Martin, 1979) while others claim the converse is true (Cockayne and Odds, 1984). Of the *Candida* species, *C. albicans* and *C. tropicalis* are best able to overcome neutrophils and escape after phagocytosis (Louria and Brayton, 1964; Fontenla De Petrino *et al.*, 1985).

The mechanisms by which *Candida* cells are killed within phagocytes have been partly elucidated. The myeloperoxidase-halide oxidative killing system is the dominant candidacidal mechanism within neutrophils (Brune *et al.*, 1973; Diamond *et al.*, 1980). Mannans can inhibit the action of the peroxidase system against the fungus, possibly by blocking binding of myeloperoxidase to the surface of yeast (Wright *et al.*, 1983).

In macrophages, as in neutrophils, oxidative and non-oxidative killing mechanisms have been demonstrated (Lehrer, 1975). Fully differentiated macrophages are more effective at killing *C. albicans* than their monocyte precursors; however, unlike monocytes, they do not have myeloperoxidase so that their oxidative killing mechanism differs from those in neutrophils and depends on the production of singlet oxygen and superoxide (Sasada and Johnston, 1980).

An antimicrobial mechanism depending on the generation of reactive nitrogen intermediates has been recently reported. The candidacidal role of this system is not

known. Although human monocytes have been reported to produce reactive nitrogen intermediates, this has not yet been demonstrated in human macrophages (Nathan and Hibbs, 1991).

1.4.2.5 Specific immune responses to *Candida*

Humoral immunity to *Candida* can be measured by detection of antibodies against the fungus. Cell mediated immunity can be measured by demonstration of DTH response in skin. The response broadly correlates with *in vitro* lymphocyte transformation tests and leukocyte migration inhibition tests.

Passive transfer into humans has provided direct evidence for the role of antibody in protection against systemic infection (Hiatt and Martin, 1946). Maiti *et al.* (1985) rendered newborn mice deficient in B lymphocytes by treating them with mouse anti-μ serum and showed that the treated mice had enhanced susceptibility to challenge with *C. albicans*. Sera from some patients with candidiasis react only with germ tubes whereas sera from normal individuals react preferentially with yeast cells (Ho *et al.*, 1979). Antibodies can function by prevention of adherence, opsonization of the organism, facilitation of cell mediated cytotoxicity and by enhancement of the function of immune lymphocytes (Diamond, 1974; Epstein *et al.*, 1981; Kagaya *et al.*, 1981; Vudhichamnong *et al.*, 1982). It has been suggested that antibodies are an important defense against secondary challenge by *C. albicans* (Ashman *et al.*, 1990).

T cells are involved in both the generation of specific immunity and resolution of primary infection. Neonatal thymectomy increased the susceptibility of mice to systemic candidiasis. This result could not be reproduced in either adult thymectomized, irradiated, bone marrow reconstituted (ATXBM) mice or in mice homozygous for the *nu* or *scid* mutations. The resistance of ATXBM and nude mice has been attributed to the activation of the monocyte/macrophage system and appears to be short-lived; in the later stages of the infection both are substantially more susceptible than control mice (reviewed by Ashman *et al.*, 1990).

Passive transfer of lymphocytes from an immune animal reduced visceral *Candida* counts in the later stages of disseminated *Candida* infection; the protective role of cell mediated immunity is important after non-specific phagocytic responses have dealt with the elimination of *Candida* in the immediate post-challenge period (Miyake *et al.*, 1977).

In systemic candidiasis, immunosuppression follows the process of uptake and/or presentation of *Candida* by macrophages and the generation of lymphocytes able to respond to the antigens. Normal human lymphocytes cultured with a polysaccharide fraction derived from *C. albicans* generate T cell suppressor factors that are able to suppress both T- and B-cell responses to the homologous antigen (Piccollela *et al.*, 1981). These effects appear to be mediated by an antigen-non-specific inhibiting factor that can block the synthesis of interleukin 2 (IL-2) and γ-inteferon as well as expression of IL-2 receptors by T cells (Lombardi *et al.*, 1986)). The factor can also inhibit antigen presentation and production of interleukin 1 (IL-1) by human monocytes (Lombardi *et al.*, 1985).

Specific immunity is also important in superficial candidiasis. Quantitative differences in cell mediated immunity, total secretory immunoglobulin levels and macrophage defects have been reported in chronic recurrent vaginal candidiasis. There was a significant correlation between the levels of anti-*Candida* IgA and IgG in serum and cervico-vaginal secretions (Gough *et al.*, 1984) and isolation of *Candida* from women of childbearing age was associated with low levels of anti-*Candida* serum IgA but not with anti-*Candida* serum IgG (Schonheyder *et al.*, 1983). Qualitative abnormalities in cell mediated immunity are associated with CMC (Kirkpatrick *et al.*, 1971).

Blood Groups and Secretor Status

After a short historical introduction, three general areas are reviewed to provide necessary background for the approach used in this study: a) the genetic control and biochemistry of blood group antigens related to secretor status; b) blood groups and disease susceptibility; c) the hypotheses proposed to explain the increased susceptibility of non-secretors to certain infectious agents.

1.5 History

In 1900, the first human blood group system was discovered by Landsteiner when he observed that red blood cells can be agglutinated by serum from another individual. Individuals could be broadly classified into four distinct groups i.e. A, B, O and AB. In 1908, Epstein and Ottenberg suggested that the ABO blood groups

were inherited (reviewed by Race and Sanger, 1975). This was proved by von Dungern and Hirzfeld in 1910; and, in 1924, Bernstein showed that the population and family data were best explained by the hypothesis that A, B and O are independent alleles at one locus (reviewed by Mourant *et al.*, 1978).

Yamakami (1926) noted that the A and B antigens were present in saliva but it was not until 1930 that it was realized by Lehrs and Putkonen that the character was dimorphic; some persons do and others do not secrete into saliva antigens corresponding to their ABO blood group. Schiff and Sasaki (1932) found that the ability to secrete antigens into body fluids behaved as a simple Mendelian factor with secretion dominant to non-secretion (reviewed by Race and Sanger, 1975).

From 1900-1927, the ABO blood group system was the only one known. The discovery of additional blood groups such as Lutheran and Kell were made in a more deliberate way: animals were injected with human red cells to stimulate antibody production. Other blood groups were discovered as a result of materno-fetal incompatibilities. In 1946, Mourant discovered the anti-Lewis^a antibodies and Andersen, in 1948, described anti-Lewis^b. It is now clear that the Lewis^a antigen (Le^a) was identified earlier in non-secretor saliva by Japanese workers. In 1951, Grubb proposed a general theory to explain the presence or absence of antigens in the saliva. Secretors have Lewis^b antigen (Le^b) and a smaller amount of Le^a whilst non-secretors have Le^a only in their body fluids (reviewed by Race and Sanger, 1975).

The ABH antigens are not confined to red cells and saliva but are found in most secretions and tissues of the human body. Phylogenetic and ontogenetic studies have established that the ABH antigens evolved earlier on epithelial cells than on blood cells; therefore, they should be considered as histo-blood group antigens (Clausen and Hakomori, 1989). The antigen now known as H is present on red cells of nearly all humans. It was long thought to be a product of the O gene; but, its nature became clearer as a result of the discovery of individuals with the Bombay blood group who lack this antigen (Bhende *et al.*, 1952, reviewed by Race and Sanger, 1975).

Population studies of blood group frequencies emphasised the differences in the distribution of the ABO phenotypes (reviewed by Race and Sanger). The function of the blood group antigens and the significance of the different frequencies in populations is not known; various hypotheses have been proposed but none have been convincingly proven (Miller, 1978; Bird, 1983; Mourant, 1983). A hypothesis on the

dual significance of ABH, Lewis and related antigens has been advanced by Le Pendu (1989). He argues that the antigens would have primarily been involved in cell-cell recognition phenomenon; and, the polymorphism would have evolved later from gene duplication under environmental pressure. The expression on erythrocytes which occurred very late in evolutionary time is probably of little biological significance; blood transfusion is an artificial situation in which the antigens on the erythrocytes are important.

1.6 ABO, Lewis Blood Group Systems and Secretor Status

1.6.1 Biochemistry of ABH antigens

The ABH-active substances are found on cells and tissues as well as in body fluids linked to lipids (glycolipids) or proteins (glycoproteins). They also exist in secretions as free oligosaccharides. Glycoproteins carry glucans in two ways. Glycosylation can occur by linking D-β-N-acetylglucosamine to the amino group of asparagine (N-linked), or by linking D-β-N-acetylgalactosamine to the oxygen group of serine/threonine (O-linked). Glycosphingolipids have β-D-glucose linked to ceramide. These lipids are further classified into lacto-, globo- and ganglioseries:

Table 1.2

(Neo)lacto-series	GlcNAcβ1→3Galβ1→ 4Glcβ1→Cer
Lactosylceramide	Galβ1→4Glcβ1→Cer
Globo-series	Galα1→Galβ1→4Glcβ1 →Cer
Ganglioseries	GalNAcβ1→Galβ1→ 4Glcβ1→Cer

Further variability is generated by the existence of various carrier carbohydrate chains that differ in branching and length. Taking into account only the terminal disaccharide, four main precursor chains are now defined (Table 1.3).

The different carrier chains show differential cell and tissue expression and core structure switching during development and differentiation (Hakomori, 1981; Oriol *et al.*, 1986; Fenderson *et al.*, 1987). Type 1 chain constitutes the main carrier of ABH antigens in body fluids and secretions. They are also expressed in endodermally

derived tissues such as lining epithelia and glandular epithelia. Generally, Type 1 chains are not found in ectodermally derived tissues although there are exceptions such as non-keratinized stratified squamous epithelium. The Type 2 chains are found mainly in ecto- and mesodermally derived tissues (Oriol *et al.*, 1986). Both Type 1 and Type 2 chains can occur as branches on a single carbohydrate chain (Lloyd *et al.*, 1968). Type 3 and 4 precursor chains have been discovered relatively recently (Takasaki *et al.*, 1978; Donald, 1981). Not much information is available with regard to their biosynthetic regulation and distribution (Breimer and Samuelsson, 1986; Clausen *et al.*; 1986).

Table 1.3

Type 1	Galβ1→3GlcNAcβ1→R
Type 2	Galβ1→4GlcNAcβ1→R
Type 3	Galβ1→3GalNAcα1→R
Type 4	Galβ1→3GalNAcβ1→R

1.6.1.1 ABH antigens on red cells

The Type 2 chain is thought to be the main carrier of the ABH antigens on red cells. The presence of Type 1 chain structure is due to absorption of circulating glycolipids from the plasma (Marcus and Cass, 1969). The Type 2 precursor chain is fucosylated by the α2 fucosyltransferase coded for by the *H* gene at the C2 position to form H Type 2 determinant (Figure 1.1). Individuals of blood groups A or B encode glycosyl-transferases capable of converting the H antigen by the addition of N-acetylgalactosamine or galactose respectively. In AB individuals the enzymes coded by these genes will compete for H Type 2; cells of these individuals will possess some molecules carrying the A determinant and others carrying the B determinant (Watkins, 1967). Both A and B determinants are not found on the same carrier molecule (Viitala *et al.*,1981). This is in contrast with saliva where A and B specificities are carried on the same macromolecules (Morgan and Watkins, 1956). Cells of an O individual express the unmodified structure of the H antigen.

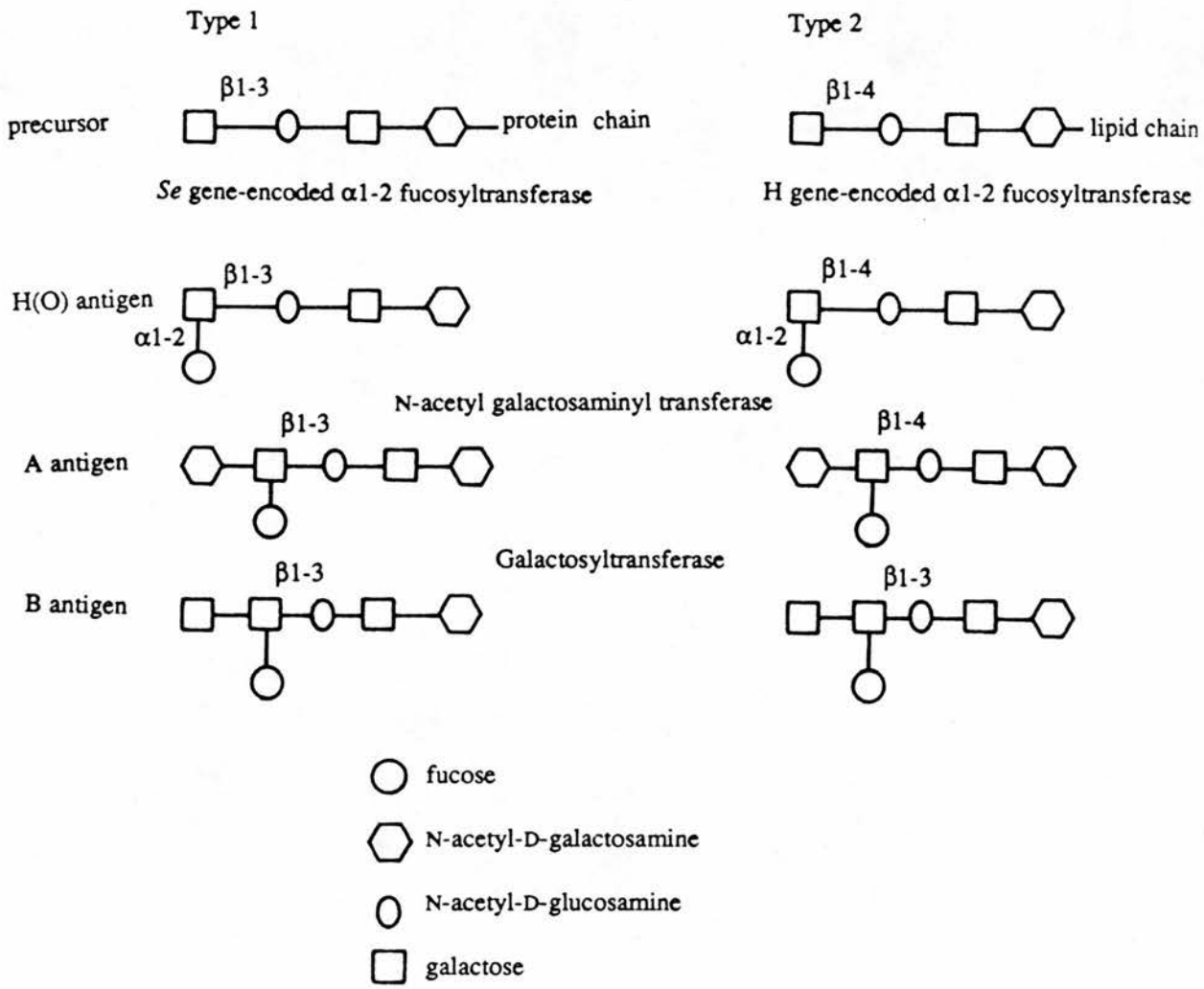


Figure 1.1 Production of A, B and H(O) antigens from precursor Type 1 and 2 chains. The enzymes facilitating conversion of antigens are shown. Note that H Type 1 and H Type 2 are substrates for N-acetyl galactosaminyl transferase and/or galactosyltransferase.

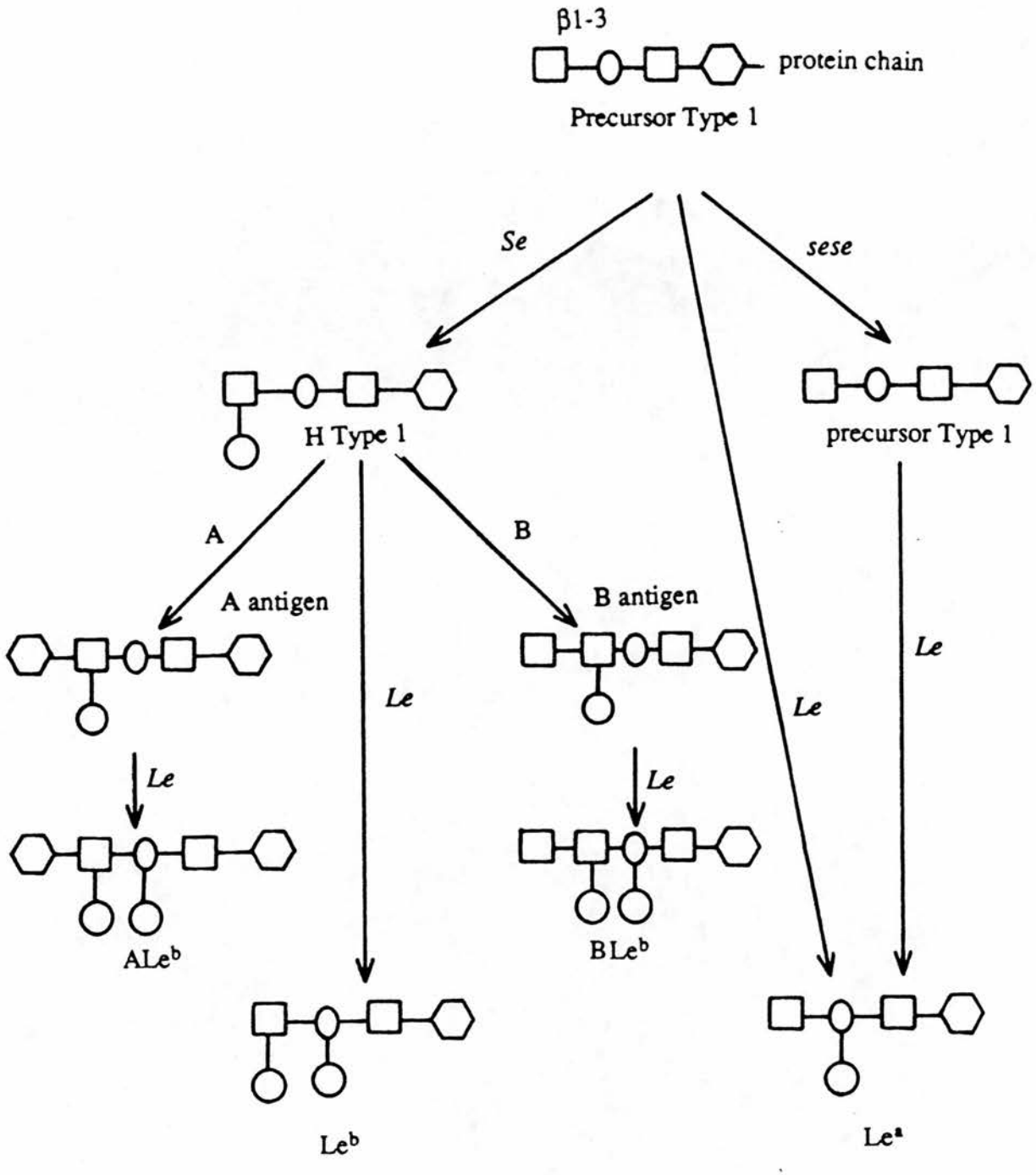


Figure 1.2 Production of H, A, B and Lewis antigens in secretions. Note that the α1-2 fucosyltransferase (*Se* gene-encoded) and the α1-4 fucosyltransferase (*Le* gene-encoded) compete for the precursor Type 1 chain. The α1-4 fucosyltransferase also competes with the A and B glycosyltransferases (see text). Symbols as in Figure 1.1.

1.6.1.2 ABH antigens in secretions

The Type 1 precursor chain is fucosylated in secretions by α1-2 fucosyltransferase coded by the secretor gene (*Se*) to form H Type 1 determinant (Watkins *et al.*, 1988). Subsequently, H Type 1 acts as a substrate to A/B glycosyltransferases to give rise to their respective determinants. The α1-2 fucosyltransferase is also able to act on Type 2 chains to a lesser degree and accounts for the presence of small amounts of H Type 2 antigens in secretions (Le Pendu *et al.*, 1982). Figure 1.1 presents the biochemical pathway by which the ABH determinants are formed from precursor Type 1 and 2 chains.

1.6.2 Biochemistry of the Lewis antigens

Unlike the ABO blood group antigens, those of the Lewis antigens do not form part of the structure of the cell membrane. They are present in the plasma and in body secretions such as saliva. In plasma, the Lewis antigens are portions of oligosaccharide chains that are bound through D-glucose to glycosphingolipids (Tilley *et al.*, 1975) and in secretions they are bound to glycoproteins (Watkins *et al.*, 1959). Once the antigens have been assembled in the plasma, they are inserted into cell membranes (Marcus and Cass, 1969).

The Lewis antigens are based on Type 1 chain structure and are generated by the α1-3/4 fucosyltransferase coded by the Lewis gene (*Le*) which adds L-fucose to N-acetyl-D-glucosamine of the precursor Type 1 chain or the H Type 1 chain to form Le^a and Le^b determinants respectively (Oriol *et al.*, 1986). The structure and formation of the Lewis antigens in secretions is shown in Figure 1.2.

The penultimate N-acetylglucosamine of Type 2 chains can, in parallel with Type 1 chains, be fucosylated to form Le^x and Le^y, isomers of Le^a and Le^b. Fucosylation of the N-acetylglucosamine in Type 2 chains has been proposed to be catalysed by an X gene-specific α1-3 fucosyltransferase specifically acting on Type 2 chains (Prieels *et al.*, 1981). In addition, it appears that the fucosyltransferase encoded by the *Le* gene can fucosylate Type 2 chains. In accordance with this view, expression of Type 2 chain structure has been found in secretions and some endodermally derived tissues to vary with the Lewis and secretor status.

Table 1.4

Gene	Chromosome	Enzyme (transferases)	Substrate
A	9	N-acetylgalactosaminyl	H Type 1 and 2
B	9	D-galactosyl	H Type 1 and 2
O	9	no product	no substrate
H	19	α1-2 fucosyl	precursor Type 2
Se	19	α1-2 fucosyl	precursor Type 1
Le	19	α3/4 fucosyl	precursor Type 1 H Type 1
X	?	α3 fucosyl	precursorType 2 H Type 2

Table 1.5 *ABO*, *Se* and *Le* genes interactions in secretions

Secretor status	Blood group antigens in body fluids				
	H*	A**	B**	Lewis^a	Lewis^b
Secretors	+	+/0	+/0	±	+
Non-secretors	-	-	-	+	0

*H is the antigen formed by the α 1-2 fucosyltransferase encoded by *Se* gene

**depending on presence of *A* and/or *B* genes

1.6.3 The *ABO*, *H*, *Se* and *Le* genes

The ABO locus present on chromosome 9 (Westerveld *et al.*, 1976) was first thought to contain three major alleles: A, B and O (reviewed by Watkins, 1980). It is now known that there are two alleles encoding for A_1 and A_2, the two subgroups of blood group A. The A and B genes differ in four single-base substitutions which change four amino acid residues. A critical single base deletion was found in the O gene which results in an entirely different inactive protein incapable of modifying the H antigen (Yamamoto *et al.*, 1990).

The term H was introduced by Morgan and Watkins (1948) to describe the antigen found in all ABO phenotypes. The *h* gene is a rare silent allele of the *H*. Individuals homozygous for *h* (*h/h*) do not have A or B antigens even if the glycosyltransferases are present; the absence of H antigen substrate renders these enzymes redundant.

The ability to produce ABH antigens in secretions is inherited in a Mendelian dominant pattern. Individuals who are homozygous or heterozygous for the *Se* gene (*Se/Se* or *Se/se*) have Type 1 blood group antigens in their body fluids. Individuals homozygous for the silent allele (*se/se*) are known as non-secretors. Among Europeans about 80% are secretors and 20% non-secretors (Race and Sanger, 1975). These percentages vary among different ethnic groups (Mourant *et al.*, 1976).

More recently, it has been proposed that the *H* gene codes an α1-2 fucosyltransferase expressed on red cells whereas the *Se* gene encodes another α1-2 fucosyltransferase expressed in salivary glands. The presence of the *Se* gene means that individuals with *h/h,Se/Se* or *h/h,Se/se* will express Type 1 blood group antigens in their secretions but will have no ABH antigens on their red cells . Such individuals are known as para-Bombay phenotype. Bombay individuals have the *h/h,se/se* genotype and fail to express ABH antigens in cells or secretions (Oriol *et al.*, 1981).

Two different forms of fucosyltransferases with the expected properties of *H* and *Se* gene products have been found by different groups to support this genetic model (Le Pendu, 1983; Kumazaki and Yoshida, 1984). It has been shown that the *H* and *Se* genes are closely linked on the short arm of chromosome 19 (Oriol *et al.*, 1986) suggesting that they might have been derived by duplication of the *Se* gene in the course of evolution (Le Pendu *et al.*, 1985).

The *Le* gene is also located on chromosome 19 but is independent of the *H* and *Se* loci (Elberg *et al.*, 1983). It encodes an α3/4 fucosyltransferase which acts on Type 1 precursor chains and, *in vitro*, on Type 2 precursor chains (Johnson *et al.*, 1981). The *X* gene encodes an α3 fucosyltransferase which exclusively uses Type 2 chains as substrates. The location of the *X* gene is not yet known; it is expressed in mesodermal tissues such as the kidney (Caillard *et al.*, 1988). Table 1.4 summarizes the *ABO*, *H*, *Se*, *Le* and *X* genes location, their enzymic product and target substrate(s) .

1.6.4 Interactions of the *H*, *Se*, *ABO* and *Le* genes

The blood group antigens are assembled through a complex mechanism whereby a series of enzymes act sequentially and specifically to extend the precursor carbohydrate chain. The specificity of the enzyme for the substrate is such that the action of one enzyme often renders the product unacceptable for another enzyme. The antigens produced thus depend on the genetic make up of the individual and the efficiency of the enzymes competing for substrates.

In secretions, the expression of the Lewis antigens depends on the secretor status (Table 1.5). When both *Se* and *Le* genes are expressed, the Type 1 precursor chain can be first fucosylated by the *Se* enzyme to produce H Type 1 antigen. This structure can be further fucosylated by the *Le* enzyme to form Le^b. If the precursor structure is first utilized by the *Le* enzyme, the structure formed (Le^a) is unsuitable as a substrate for the *Se* encoded enzyme. As a result, both Le^a and Le^b are formed in secretors; the relative amounts found depend on the efficiency of the competing enzymes (Watkins *et al.*, 1988; Ogata *et al.*, 1988). The A and B enzymes also compete with the Lewis enzymes for the same substrate (H Type 1). Once the determinant has been converted to Le^b, it cannot function as a substrate for the A and B transferases. On the other hand, A and B determinants can be further fucosylated by the enzyme coded by the *Le* gene to form ALe^b or BLe^b determinants.

In non-secretors, the absence of the *Se* encoded enzyme means that the only determinant formed from the Type 1 precursor chain is Le^a. In the absence of both *Se* and *Le* genes, secretions will contain unconverted Type 1 precursor chain structures.

In tissues the interaction of the genes can be generalized as follows: ectodermal tissues express ABH antigens independent of the *Se* gene although they can be quantitatively influenced by the *Se* gene (Dabelsteen *et al.*, 1982); endodermal tissues

express ABH and Lewis antigens under the control of the products of the *Le* and *Se* genes (Oriol, 1987).

1.7 Blood Group and Disease Susceptibility

Following the discovery that blood group frequencies differed in various populations, the obvious question is "What are the origins of these differences?". There are two possibilities: genetic drift and natural selection. Both processes are thought to be operative, but their relative importance is unclear.

The founder effect due to migration of a small non-representative group of individuals might be responsible for an initial difference in gene frequencies between the main and migrant populations. Genetic drift could then affect the migrant population so that it becomes progressively more different from the main population.

Natural selection arises from differences in fitness between individuals with various blood groups. If a phenotype is at a disadvantage, this might result in the environment causing a raised early mortality or a lowered fertility in persons with the particular phenotype compared with those of another phenotype.

One of the outstanding examples of association between blood group and early mortality is that involving haemolytic disease of the newborn (Levine *et al.*, 1941). This is the result of anti-Rhesus antibodies which develop in a Rhesus-negative mother carrying a Rhesus-positive foetus. A similar phenomenon exists with ABO incompatibility of mother and foetus (Halbrecht, 1944). There is a marked deficiency of ABH non-secretors among infants suffering from ABO haemolytic disease compared with the general population (Levene and Rosenfield, 1961).

Incompatibility of the ABO blood group and secretor status are also thought to be important causes of early abortions, infertility and still births. There is an excess of spontaneous abortions (42.6% compared with 35% expected) with the ABO incompatible parents (Levene and Rosenfield, 1961). The association between ABO blood group and infertility has been suggested on the basis of a study in which there was a significant excess of ABO incompatible couples (87.3%) in persistently sterile matings compared with 38.6% in fertile couples (Behrman *et al.*, 1960) These authors suggest that ABO related infertility is due to the action of antibodies in the secretions of the mother's genital tract on incompatible spermatozoa. Two other studies found a

similar trend but the results were not significant. There is, therefore, evidence that in ABO incompatible matings, there is a marked selection against the birth or survival of the ABO incompatible foetus and that non-secretors are favoured *in utero*.

Early mortality could also be influenced by susceptibility to infectious agents. Disease due to bacteria, viruses, protozoa and fungi have been studied for associations with blood group and with secretor status in some cases. Selected examples are described below and summarized in Table 1.6.

1 Protozoa

Malaria is an outstanding example of an infection influencing the genetic frequency within a population. Only quartan malaria (*Plasmodium vivax*) and malignant/tertian malaria (*Plasmodium falciparium*) have been studied. The disease has strong associations with several polymorphic genes such as the sickle cell gene, the Duffy blood group, β-thalassemia (reviewed by Mourant *et al.*, 1978), HLA Bw26 and DQ/DR haplotypes (Hill *et al.*, 1991). The presence of sickle cell gene and the HLA haplotypes is protective against *severe* malaria infection; these associations are not significant among patients with mild or moderate disease. Associations between ABO blood group and disease await further extensive surveys which differentiate the type of parasite, the vector as well as severity of the infection. When both forms of malaria are considered, the data show a marked excess of A patients compared with O and B individuals. Athreya and Corriell (1967) have suggested that blood group B confers a selective advantage in relation to malaria infection to explain the high frequency of blood group B corresponding to where malaria is endemic.

2 Bacteria

One of the earliest observation of particular blood groups and secretor status associated with bacterial disease was that by Glynn *et al.* (1956) (reviewed by Haverkorn and Goslings, 1969). Scarlet fever is caused by Group A β haemolytic *Streptococcus pyogenes*. Carriers of the bacteria show a consistent deficiency of ABH secretors but no blood group bias (Haverkorn and Goslings, 1969). Scarlet fever patients, however, show a significant excess of blood group O individuals (Mourant *et al.*, 1978). It appears that non-secretors are prone to carriage; and, of these, blood group O individuals might develop disease.

Table 1.6 Associations between blood group and/or secretor status and susceptibility to infections

Infectious agent	Secretor status association	Blood group associations
Urinary Tract		
Escherichia coli	non-secretors	B
Gastrointestinal Tract		
Vibrio cholerae	non-secretors	O
Genitourinary Tract		
Neisseria gonorrhea	none	B or none
Chlamydia trachomatis	non-secretors	B
HIV (heterosexual transmission)	secretors	?
Respiratory Tract		
Respiratory Syncytial Virus	secretors	?
Rhino virus	secretors	?
Echo virus	none	?
Influenza A	secretors	O
Parainfluenza virus	none	?
Streptococcus pyogenes (Group A)	non-secretors	not O
Streptococcus pneumoniae	non-secretors	not B
Neisseria meningitidis	non-secretors	none
Haemophilus influenzae type b	non-secretors	?
Oral cavity		
Candida albicans carriage	non-secretors	O
Candidiasis	non-secretors	none
Caries	non-secretors	?
Periodontal disease	none	O and AB

Table reproduced from Blackwell (1989) with modifications.
? indicates lack of information in the literature

Over the last decade, other diseases caused by bacteria have been found to be associated with secretor status. Non-secretors are prone to invasive disease due to *Neisseria meningitidis*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, urinary tract infections due to *Escherichia coli* and cholera (reviewed by Blackwell, 1989).

3 Viruses

Among viral diseases, two influenza A viruses appear to infect more blood group O individuals than A or B (McDonald and Zuckerman, 1962). Examination of sera from survivors of past epidemics shows only a slight and non-significant increase of group O individuals among strong reactors for each of the two different influenza A virus strains (Watkin *et al.*, 1975). Both Cuadrado and Davenport (1970) and Otter and Schild (1967) found an increase in group O individuals among positive reactors (reviewed by Mourant *et al.*, 1978). A recent study of upper respiratory infection viruses reported a significant increase of secretors of ABH antigens among patients with acute infections from whom viruses were isolated (Raza *et al.*, 1991). Secretors are also over-represented among individuals who have acquired human immunodeficiency virus (HIV) through heterosexual intercourse (Blackwell *et al.*, 1991).

1.7.1 Blood groups and other associations

Apart from infectious diseases, secretor status has been associated with other diseases for which infectious triggers have been proposed, e.g. Graves' disease, insulin dependent diabetes mellitus and peptic ulcers (Blackwell, 1989; Mourant *et al.*, 1978). Blood groups have also been associated with other conditions such as neoplasms, thromboembolic and developmental disorders (Mourant *et al.*, 1978). These associations might influence mortality but will not be discussed here.

1.7.2 Mechanisms by which blood group and secretor status influence disease susceptibility

Genetic susceptibility to infectious diseases has been accepted for many years. The mechanisms underlying the genetic susceptibility are obscure in most cases. Theoretically, genes can exert their influence directly (through the encoded product) or indirectly via modification of end-products encoded by other genes. For example,

although the association between HLA and various diseases has been known for several decades, researchers have concluded that genes at other loci contribute to the overall susceptibility of an individual to disease (Ryder *et al.*, 1981). The following are hypotheses suggested to explain associations between susceptibility to infectious agents and blood group or secretor status.

1.7.2.1 Secretor status and complement system

Although components of the complement system are encoded by genes on chromosome 6, the gene that codes for the C3 component is in the same linkage group as the *Se* gene on chromosome 19 (Elberg *et al.*, 1983). Compared with healthy individuals, C3 levels are lower among individuals with insulin-dependent diabetes mellitus (IDDM) but not those with non-insulin dependent diabetes mellitus (NIDDM) (Charlesworth *et al.*, 1987). These two reports together with the observation that non-secretors are over-represented among individuals with IDDM (Blackwell *et al.*, 1987a) promoted a study of C3 and C4 levels among patients with IDDM with respect to secretor status. Significantly lower levels of C3 and C4 were found among individuals with IDDM who were non-secretors compared with those who were secretors (Blackwell *et al.*, 1988).

The mechanism whereby lower C3 levels are found in non-secretors and its effect on disease susceptibility is not clear. The reduced C3 levels are not due to a more rapid clearance of complement factors, exhaustion of complement factors, or attachment to immune complexes (Charlesworth *et al.*, 1987).

Furthermore, the gene that codes for C3 exhibits genetic polymorphism (Alper, 1986). The alleles are inherited in an autosomal co-dominant manner. Several C3 variants have been found and characterised by agarose gel electrophoresis. Only two forms occur frequently i.e. C3F (fast) and C3S (slow). The gene frequency of C3S ranges between 77%-99% depending on the population sampled.

The biological significance of C3 polymorphism is not known. Several studies have reported a significant increase of C3F allele among patients with chronic inflammatory diseases such as rheumatoid arthritis (Bronnestam, 1973), Crohn's disease (Elmgreen *et al.*, 1984) and autoimmune diseases such as multiple sclerosis (Jans and Sorensen, 1980) and cirrhosis (Srivastave and Srivastave, 1985). The C3F protein exhibits increased capacity for binding onto receptors of mononuclear cells compared with the C3S protein (Arvillomi, 1974) suggesting that the functionally

active site of the molecules differ. A relationship between C3S and high titres of antibodies to A and B blood group antigens in mothers with ABO incompatible foetuses has been reported (Bronnestam and Cedegren, 1973); and, these authors suggest that the C3S variant might influence antibody production through unknown mechanisms. Since the *Se* and *C3* genes are linked, it is possible that the inheritance of *se* along with the *C3F* allele could account for the increased susceptibility of non-secretors to disease.

1.7.2.2 Secretor status and immunoglobulins

The only hypothesis proposed to explain the association between blood groups and susceptibility to disease was that anti-A and anti-B isohaemagglutinins acted as "natural" antibodies against microorganisms expressing antigens cross-reacting with A or B (Springer *et al.*, 1961). Grundbacher and Scheffler (1970) reported a significantly higher level of B isoantibodies in the serum of secretors compared with non-secretors. These authors extended their studies by examining serum IgG levels of anti-B and found them to be higher among secretors compared with non-secretors. Subsequently, studies of total serum and salivary IgA levels and total serum IgG were shown to be higher among secretors compared with non-secretors (Waissbluth and Langman, 1971; Grundbacher, 1972).

Studies by Blackwell and colleagues however, have not demonstrated this trend. Among women recently referred for assessment of recurrent urinary tract infections (UTI), the total serum IgA and IgG levels were found to be similar for secretors and non-secretors; however, among long term sufferers of UTI, non-secretors had significantly higher titres of total serum IgA and IgG than secretors (Blackwell *et al.*, 1987b).

In the Stonehouse survey for carriage of *Neisseria meningitidis*, carriers had significantly higher levels of total salivary IgA compared with non-carriers. Among non-carriers, basal salivary IgA levels were similar among secretors and non-secretors; however, total serum IgA was significantly higher among secretors compared with non-secretors. No difference in total serum and salivary IgA was found between secretors and non-secretors among carriers of *N. meningitidis* (Blackwell *et al.*, 1989b). Similarly, among patients with spondyloarthropathies no difference in total serum and salivary IgA was found between secretors and non-secretors (Shinebaum *et al.*, 1987).

Table 1.7 Blood group antigens that are receptors for microorganisms

Microorganisms	Blood group receptors
Escherichia coli	
Uropathogenic strain	P M N
Septicaemia and neonatal meningitis	S
H. influenzae type b	Anton
Candida	Lewis[a]
Plasmodium knowlesi	Duffy

Recent work on antibodies specific for *Neisseria lactamica* and *N. meningitidis* isolates found that salivary IgM antibodies are significantly elevated among secretors compared with non-secretors (Zorgani *et al.*, 1992). Infants are dependent on secretory IgM to protect mucosal surfaces since very little secretory IgA is present (Melande *et al.*, 1984). This might explain the increased susceptibility of non-secretors to colonization and disease by *Neisseria spp* (Blackwell *et al.*, 1989b). This study emphasises the importance of recording specific antibodies against a known infecting agent in future studies on the role of secretor status in humoral immunity.

1.7.2.3 ABH and Lewis antigens as receptors for infectious agents

The susceptibility of individuals of certain blood groups to infectious agents has been explored in terms of variability of attachment of microorganisms to host cells expressing different blood groups. Table 1.7 lists examples of blood group antigens that act as receptors for microorganisms (Blackwell, 1989). The best example is the Duffy blood group antigens and susceptibility to *Plasmodium vivax.*

The Duffy alleles Fy^a and Fy^b are associated with susceptibility to *P. vivax* (Miller *et al.*, 1975). Africans and American Blacks do not have the Duffy antigens (Fy^{a-},Fy^{b-}) and are resistant to these infections; non-African populations possess either or both of these antigens. *Plasmodium knowlesi* is closely related to *P. vivax* and has been used for *in vitro* studies which showed that erythrocytes with Fy^{a+b-}, Fy^{a-b+} and Fy^{a+b+} phenotypes were invaded equally by *P. knowlesi.* Almost no invasion was observed for Duffy negative erythrocytes (Fy^{a-b-}). When the Duffy antigens were removed by proteolysis, the erythrocytes became resistant to invasion. Subsequent studies showed that the Duffy determinant Fy^6 is a receptor for *P. vivax* and *P. knowlesi* merozoites (Barnwell *et al.*, 1989). This structure is expressed on all human erythrocytes except those from individuals with Fy^{a-b-} genotype (Nichols *et al.*, 1987). The erythrocyte receptors for *P. falciparum* are probably glycophorin A and an anion transport protein (Wallach, 1986). The resistance to invasion by *P. vivax* of Duffy negative individuals compared with the other Duffy blood groups was confirmed in a clinical study using human subjects (Miller *et al.*, 1976).

Non-secretors of the ABH antigens are at an increased risk of superficial disease due to *Candida* (Thom *et al.*, 1989) and carriage of these yeasts (Burford-Mason *et al.*, 1988). Fucose has been identified as a receptor for *C. albicans* isolated from oral

lesions. Fucose is the immunodominant sugar of the H and Lewis blood group antigens. Two hypothesis were proposed to explain the increased susceptibility of non-secretors to *C. albicans*. The first is that the H and Le^b antigens in the body fluids of secretors might act to block the attachment of *Candida* to epithelial cells. This was supported in a study which showed that *Candida* treated with boiled secretor saliva were inhibited from binding to buccal epithelial cells (BEC) from non-secretors. Yeasts treated with boiled non-secretor saliva, however, bound in greater numbers to BEC of both secretors and non-secretors compared with untreated blastospores (Thom *et al.*, 1989). This gave rise to the second hypothesis: Le^a antigen found in greater quantities on cells and in saliva of non-secretors might act as a receptor which promotes binding of yeasts to BEC. Support for this hypothesis was provided by a study in which binding of *Candida* to BEC from non-secretors was inhibited by polyclonal anti-Le^a antibody; treatment of BEC from secretors with either anti-Le^a or anti-Le^b did not inhibit binding of the blastospores (May *et al.*, 1989).

1.8 Aims of the study

Oral candidiasis affects individuals whose host defences are compromised. Patients with diabetes were chosen as a sample population because they represent one of the immunocompromised groups who are susceptible to candidiasis and can be used as a model for other immunocompromised groups such as individuals with HIV or those undergoing immunosuppresive therapy. Patients with diabetes are a large population who are easily accessible and relatively safe to sample compared with individuals with HIV infection. The aims of the study were two fold:

1. to define the role of the secretor gene and other risk factors in oral carriage of *Candida* species and in development of denture stomatitis among individuals with insulin dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM).

2. to investigate the factors underlying the increased susceptibility of non-secretors to oral disease and carriage of yeasts, particularly the role of secretor status in adherence of *C. albicans* to epithelial cells.

Chapter 2
Carriage of Yeasts

2.1 Introduction

Oral candidiasis is most prevalent as chronic atrophic candidiasis (Odds, 1988). Individuals with diabetes are prone to infection; and, chronic atrophic candidiasis has been reported to be more prevalent among diabetic individuals compared with non-diabetic controls (Lamey *et al.*, 1988). Since disease is frequently preceded by colonization, it is important to identify factors which predispose to colonization. Few studies have examined the factors which predispose to colonization of diabetic patients by *C. albicans*; and, none of these specifically analysed patients with IDDM separately from those with NIDDM (Barlow and Chattaway, 1969; Tapper-Jones *et al.*, 1981; Fisher *et al.*, 1987; Lamey *et al.*, 1988; Hill *et al.*, 1989).

Both genetic (Burford-Mason *et al.*, 1988) and environmental factors (Ryley, 1986) have been reported to affect carriage of *Candida* species. Group A β haemolytic *Streptococcus pyogenes* are isolated more frequently from the pharynx of individuals who are non-secretors of ABH antigens (Haverkorn and Goslings, 1969) and non-secretors were significantly over-represented among carriers of meningococci (Blackwell *et al.*, 1990). Among non-diabetic individuals and individuals with NIDDM non-secretors of blood group O are over-represented among carriers of *C. albicans* compared with secretors (Burford-Mason *et al.*, 1988; Blackwell *et al.*, 1989a).

Environmental factors variably reported to be associated with carriage of yeasts include: presence of a denture; continuous wearing of a denture; denture fit, occlusion, trauma, hygiene and age; smoking; and control of diabetes (Budtz-Jorgensen and Bertram, 1970; Tapper-Jones *et al.*, 1981).

This was the first study designed to compare the factors contributing to carriage of yeasts in individuals with IDDM or NIDDM; its aims were:

1. to compare the oral rinse technique with the palate swab method for isolation of yeasts.

2. to compare the species of yeasts isolated from patients with IDDM or NIDDM.

3. to assess the association between secretor status and carriage of yeasts among individuals with IDDM or NIDDM taking into account denture status.

4. to dissect the contributions of the following variables to the carriage of yeasts and, specifically, *C. albicans*: age; gender; type of diabetes; control of diabetes as measured by glycosylated haemoglobin A_1 (HbA_1); random plasma glucose levels; persistent glycosuria and albuminuria; diabetic complications - retinopathy, neuropathy, nephropathy; antibiotic usage; corticosteroid treatment; smoking; alcohol consumption; presence of the denture in the mouth at night; denture fit, extension, occlusion, hygiene and age; presence of denture stomatitis; and history of superficial candida infections.

2.2 Materials and Methods

2.2.1 Subjects

A total of 439 subjects attending for routine follow up examination at the Diabetic Outpatients' Department (DOPD), Royal Infirmary, Edinburgh were sampled. An initial pilot study examined 80 individuals and was followed by a study that sampled 359 individuals between September 1988 and March 1989. The method of selection was stratified random selection according to gender and type of diabetes.

2.2.2 Clinical history

Each subject was classified as insulin dependent (IDDM) or non-insulin dependent (NIDDM) according to family history of diabetes, clinical history of onset, requirement for insulin and progression of the disease. Of the 439 subjects sampled, three could not be classified.

A full medical history including the presence of diabetic complications (retinopathy, neuropathy and nephropathy) was obtained during interview and from the patients' records. A history of medications, with particular reference to antibiotics or corticosteroid-containing preparations, within the past 6 months was noted. A social history of alcohol consumption and smoking was recorded. Subjects were questioned about history of superficial infections due to yeasts. Glycosuria and albuminuria were recorded as persistent if subjects had positive urine samples on

more than two consecutive appointments at the DOPD. None of the subjects used any oral preparations containing antiseptics within the previous 6 months.

2.2.3 Clinical examination

A thorough oral examination of both soft and hard tissues was carried out. Any of the following abnormalities were noted: angular cheilitis; leukoplakia; median rhomboid glossitis; fissured, geographic or hairy tongue; and denture stomatitis. The occlusion, fit, extension and hygiene of a denture where present was recorded as "good" or "poor". The age of the denture was recorded as well as whether it was left out of the mouth at night.

2.2.4 Samples

Venous blood was obtained for ABO blood grouping and Lewis antigen determination. Routine analyses for glycosylated haemoglobin (HbA_1) and random plasma glucose were recorded. The Corning electrophoresis method was used for measuring HbA_1 (normal range 4.5-8.0%, Coefficient of variance = 4%).

Swabs were obtained from five sites of the mouth including the palate and inoculated immediately into malt broth. Each subject provided a fresh, unstimulated sample of saliva which was collected in a sterile Universal container.

Subjects were requested to rinse with 10 mls of sterile phosphate buffered saline (PBS) for 1 minute and to return the contents to a sterile Universal container.

2.2.5 Laboratory analysis

ABO blood group was determined by slide agglutination with monoclonal anti-A and anti-B antibodies (Scottish National Blood Transfusion Service). Secretor status was determined by the presence of Lewis antigen on red blood cells by the tube agglutination with monoclonal anti-Le^a and anti-Le^b antibodies (Scottish National Blood Transfusion Service). Saliva was boiled within 1 hr of collection and stored at -20 °C. The haemagglutination inhibition method with boiled saliva was used to confirm the Lewis antigen results for 159 individuals (Mollison, 1983).

The swabs in malt broth were incubated at 37 °C for 36-48 hr, plated onto malt

agar and incubated for a further 36-48 hr.

The mouth rinse was concentrated by centrifugation and resuspended in 1 ml of PBS, 20 ul of the suspension were inoculated onto malt agar plates and incubated at 37 °C for 36-48 hr. The number of colonies per sample was recorded.

Pure colonies were subcultured and identified with the API 20 C Auxanogram (API Systems S.A, France). All were also identified by the conventional methods of germ tube production in horse serum, urease test and hyphae production on corn meal agar following incubation at 28 °C for 48 hr (Milne, 1989).

2.2.6 Statistical analysis

All results were coded and a computerised database was set up to facilitate analysis by the SPSSX statistical programme. Univariate analysis was by chi-square (with Yates' correction) or Wilcoxon rank sum tests. MacNemar's test for paired alternatives was used to compare the results of the oral rinse and the palate swab.

Stepwise linear discriminant analysis (Wilk's method) was used to identify which combinations of factors best predicted yeast carriage. The grouping variable is a binary variable i.e. carrier or non-carrier. The programme selects the linear combination of variables that best predicts the grouping variable. During the analysis, predictor variables can be added or removed in a stepwise manner, each variable being selected on the basis of an F-level of 4 for entry or removal, corresponding to $p = 0.05$.

2.3 Results

Table 2.1 summarizes the characteristics of the population sampled with respect to their diabetic status. Individuals with IDDM were younger, had a higher mean value of HbA_1; and, a significantly higher proportion of non-secretors (36.4%) compared with individuals with NIDDM (22%)($\chi^2 = 10.15$; $p < 0.005$). The mean number of cigarettes per day smoked by subjects with IDDM or NIDDM were similar.

Some problem - multiple species ? Did you find different yeasts at different sites in the same individual ??

2.3.1 Mycological profile of the sample population

Only 29% (128/439) of the individuals examined had no yeasts in any of the five sites swabbed; this compared well with the oral rinse technique in which 34% (144/439) of the individuals were culture negative. The concordance between the results obtained by these methods was 85%. By MacNemar's test for paired alternatives, there was no significant difference between the discordant pairs obtained by the two sampling methods ($p > 0.1$). Table 2.2 compares the species of yeasts cultured from swabs of the five sites of the mouth with those obtained from the oral rinse from individuals with IDDM or NIDDM. *T. glabrata* and *C. tropicalis* were isolated more frequently from those with dentures. Yeasts were isolated significantly more frequently from the palate of individuals with IDDM compared with those with NIDDM (Table 2.3); similar results were obtained with the oral rinse method.

2.3.2 Univariate analysis

Isolation of yeasts from the palate or from the oral rinse was not associated with secretor status when the results were analyzed by type of diabetes and/or denture status.

Blood group was not associated with palatal carriage. When carriage was assessed by the oral rinse, however, individuals with NIDDM who wore dentures and who were of O blood group were more likely to be carriers than those of blood group A ($\chi^2 = 7.93$, $p = 0.005$) (Table 2.4). Comparisons with B and AB were not done because of the small numbers of individuals with these blood groups. Analysis of secretor status with reference to blood group revealed a significant association between non-secretion and increased frequency of carriage of yeasts among individuals with IDDM who were blood group A and who wore dentures (9/19 A-secretor carriers compared with 12/12 A-non-secretors carriers $\chi^2 = 7.07$, $p = 0.008$).

Tables 2.4 to 2.8 present analysis of factors examined and carriage of yeasts; similar results were obtained when carriage of *C. albicans* was analysed separately.

Increase in age was associated with a decreased frequency of isolation of yeasts from the palate (Table 2.5). Similar results were obtained by the oral rinse technique.

The HbA_1 values of palatal carriers were higher compared with non-carriers

Table 2.1 Characteristics of the sample population

Type of diabetes (no.)	Age mean (sd)	HbA_1 mean (sd)	Smokers (%)	cigarettes per day mean(sd)	non-secretors (%)	secretors (%)
IDDM (231)	40.3 (16.0)	10.3 (2.1)	41	17.3 (11.1)	36.4	63.6
NIDDM (205)	58.1 (9.3)	9.8 (2.3)	34	17.3 (11.7)	22.0	78.0

Table 2.2 Species of yeast isolated from individuals with IDDM or NIDDM with or without dentures (figures represent percentages).

Patient category	Sample	none	*C. albicans*	*C. tropicalis*	*C. glabrata*	Other
No dentures						
IDDM						
(n = 150)	PBS oral rinse	33	47	2	0	18
	Palate swab	44	48	1	1	6
	Tongue swab	38	54	2	0	6
	Floor of mouth swab	41	54	1	1	3
	Right angle swab	56	38	0	0	5
	Left angle swab	60	35	0	0	5
NIDDM	PBS oral rinse	40	28	2	0	31
(n=70)	Palate swab	63	28	0	0	9
	Tongue swab	63	32	0	0	5
	Floor of mouth swab	70	27	0	0	3
	Right angle swab	68	27	0	0	5
	Left angle swab	75	24	0	0	2
Dentures						
IDDM						
(n = 79)	PBS oral rinse	24	43	4	0	29
	Palate swab	33	47	9	2	9
	Tongue swab	37	49	5	2	8
	Floor of mouth swab	34	49	5	3	8
	Right angle swab	50	29	9	1	10
	Left angle swab	42	41	6	2	9
NIDDM	PBS oral rinse	41	24	6	3	27
(n=135)	Palate swab	51	29	6	3	12
	Tongue swab	51	34	8	2	6
	Floor of mouth swab	53	31	7	3	7
	Right angle swab	54	25	9	3	9
	Left angle swab	58	24	10	3	6

Table 2.3 Type of diabetes and isolation of yeasts from the palate swab

Patient Category	Type of diabetes	non-carriers no.	(%)	carriers* no	(%)	χ^2	p
All	IDDM	70	(39)	108	(61)	8.5	<0.01
	NIDDM	101	(55)	82	(45)		
no dentures	IDDM	48	(44)	62	(56)	5.03	0.01
	NIDDM	40	(62)	24	(38)		
dentures	IDDM	22	(33)	44	(67)	4.81	0.01
	NIDDM	61	(51)	58	(49)		

*All yeasts including *C. albicans*
p value refers to comparisons between IDDM and NIDDM

Table 2.4 Blood groups and carriage of yeasts assessed by oral rinse technique

Category	Blood group	Non-carriers no.	(%)	carriers no.	(%)	χ^2	p
IDDM	O	22	(39)	34	(61)	1.49	0.22
no dentures	A	11	(26)	32	(74)		
IDDM	O	5	(16)	26	(84)	1.24	0.27
dentures	A	10	(31)	22	(69)		
NIDDM	O	9	(33)	18	(67)	0.13	0.72
no dentures	A	7	(44)	9	(56)		
NIDDM	O	17	(32)	36	(68)	7.93	<0.01
dentures	A	20	(67)	10	(33)		

p refers to comparison of carriage between individuals of blood group O with those of blood group A.

Table 2.5 Median age and carriage of yeasts determined by palate swab

Patient Category	Median age (years)		p
	non-carriers	carriers	
IDDM			
dentures	58.00	49.00	0.03
(n)	(22)	(44)	
no dentures	38.50	29.00	0.01
(n)	(48)	(62)	
NIDDM			
dentures	61.00	59.00	0.07
(n)	(61)	(58)	
no dentures	56.00	50.50	0.09
(n)	(39)	(24)	

p determined by Mann-Whitney test

Table 2.6 Glycaemic control (HbA_1) and carriage determined by palate swab

Patient Category	Median HbA_1 units		p
	non-carriers	carriers	
IDDM			
dentures	9.55	11.10	0.067
(n)	(18)	(236)	
no dentures	9.30	10.60	0.017
(n)	(45)	(55)	
NIDDM			
dentures	9.30	9.50	0.15
(n)	(57)	(52)	
no dentures	8.70	9.90	0.26
(n)	(40)	(21)	

p determined by Mann-Whitney test

Table 2.7 Smoking and carriage of yeasts determined by palate swab

Category	Mean cigarettes/day		p
	non-carriers	carriers	
ALL	4.54	7.83	<0.01
(n)	(156)	(181)	
IDDM			
dentures	7.95	9.20	0.24
(n)	(20)	(44)	
no dentures	5.72	7.32	0.43
(n)	(43)	(59)	
NIDDM			
dentures	2.89	6.87	0.05
(n)	(57)	(54)	
no dentures	4.28	9.09	0.13
(n)	(36)	(23)	

p determined by Mann-Whitney Test

Table 2.8 Continuous wearing of denture and isolation of yeasts from the palate

Patient Category	denture present	non-carriers no	(%)	carriers no	(%)	χ^2	p
All	Yes	30	(40)	45	(60)	6.61	0.01
	No	32	(65)	17	(35)		
IDDM	Yes	10	(32)	21	(68)	0.00	1.00
	No	5	(38)	8	(62)		
NIDDM	Yes	20	(45)	24	(55)	5.97	0.015
	No	27	(75)	9	(25)		

p value refers to comparison between carriage and the presence or absence of the denture at night

Table 2.9 Palatal carriage of yeasts: variables isolated by multivariate analysis (Wilk's Method)

Category	Variables isolated	% cases correctly predicted
IDDM		
no dentures (n = 110)	Age ($p < 0.05$)	61%
dentures (n = 51)	HbA_1 ($p < 0.01$) Age ($p < 0.05$) Plasma glucose ($p < 0.05$)	71%
NIDDM		
no dentures (n = 49)	Glycosuria ($p < 0.01$)	78%
dentures (n = 80)	Continuous wear of denture ($p < 0.01$)	64%

Table 2.10 Oral carriage of yeasts: variables isolated by the multivariate analysis (Wilk's Method).

Category	Variables isolated	% cases correctly predicted
IDDM		
no dentures (n = 97)	Age ($p < 0.01$)	68%
dentures (n = 71)	Retinopathy ($p < 0.05$)	76%
NIDDM		
no dentures (n = 43)	Glycosuria ($p < 0.01$) plasma glucose($p < 0.05$) non-secretion ($p < 0.05$)	67%
dentures (n = 78)	plasma glucose ($p < 0.05$)	48%

particularly among individuals with IDDM (Table 2.6). Results from the oral rinse technique showed a similar trend but did not attain significance at $p \leq 0.05$ for individuals with IDDM or NIDDM.

The mean number of cigarettes per day was a highly significant factor associated with carriage of the whole population sampled (Mann-Whitney $p < 0.001$). When results were analysed with respect to type of diabetes and denture status, smoking was of marginal significance ($p = 0.051$) only among denture wearers with IDDM (Table 2.7).

Yeasts were isolated more often from the palate of subjects who left their dentures in the mouth at night; however, this was observed only among individuals with NIDDM (Table 2.8). This pattern was not found when carriage was assessed by the oral rinse method.

The following variables were not associated with frequency of carriage of yeasts by either isolation technique: gender; duration of diabetes; complications - neuropathy, nephropathy and retinopathy; alcohol consumption; contraceptive pill; systemic corticosteroid treatment; topical corticosteroid application; antibiotics; fit, occlusion, age or hygiene of denture; persistent albuminurea; history of superficial *Candida* infections. Denture status was not significantly associated with frequency or density of colonization by yeasts.

2.3.3 Multivariate analysis

Univariate analysis might incorporate dependent variables which need prior knowledge to control for their effect. The multivariate analysis identified factors which contribute to carriage of yeasts among individuals with IDDM or NIDDM who wear dentures and those without dentures. Tables 2.9 and 2.10 summarize the results obtained from the palate swab and oral rinse techniques; similar results were obtained when carriage of *C. albicans* was analysed separately. The percentage of carriers correctly predicted by the isolated variables indicates their prognostic value.

Individuals with IDDM who did not wear dentures were best segregated on the basis of their age into non-carriers and carriers of yeasts; carriage decreased with increased age. This was true when carriage was assessed by either technique (Tables 2.9 and 2.10). The univariate analysis showed similar associations; younger individuals were more prone to carriage of yeasts (Table 2.5).

Among individuals with IDDM who wore dentures, an increase in the frequency of palatal carriage of yeasts was associated with an increase in HbA_1 level ($p < 0.01$); younger individuals ($p < 0.05$) and increased random plasma glucose levels ($p < 0.05$) (Table 2.9). The variable which was the most efficient predictor of carriage when assessed by the oral rinse technique was, however, the presence of retinopathy ($p < 0.05$) (Table 2.10). Univariate analysis of this category showed age to be associated with palatal carriage (Table 2.5).

Individuals with NIDDM who did not wear dentures were more likely to be palatal carriers of yeasts if they had persistent glycosuria ($p < 0.05$) (Table 2.9). Carriage assessed by the oral rinse method was influenced by persistent glycosuria ($p < 0.01$), higher random plasma glucose levels ($p < 0.05$) and non-secretion of blood group antigens ($p = 0.05$) (Table 2.10). Univariate analysis revealed an association between persistent glycosuria and carriage among individuals with NIDDM ($\chi^2 = 5.32$; $p = 0.02$).

Individuals with NIDDM who wore dentures were at risk of palatal carriage of yeasts if they wore their dentures continuously ($p < 0.01$) (Table 2.9). Carriage of yeasts as assessed by the oral rinse technique was best predicted by an increased random plasma glucose levels ($p < 0.05$) (Table 2.10). Comparison with univariate analysis showed that continuous wearing of dentures was a significant factor only when carriage was assessed by palatal swab (Table 2.8).

2.4 Discussion

The results are discussed in the context of the objectives of the study. The first objective of the study was to compare the rate of isolation of yeasts by the oral rinse technique with that obtained by the palate swab. The oral rinse technique compared well with the results of the swab technique; there was 85% concordance between the results obtained with the two methods.

C. albicans was the species most frequently isolated from the swabs while species other than *C. albicans* were isolated more often from the oral rinse. This might be due to incubation of swabs in the malt broth suppressing other species of yeasts. By the swab technique, yeasts were isolated most frequently from the tongue followed by the palate, floor of the mouth and the angles of the mouth. Similar results have been reported (Tapper-Jones *et al.*, 1981). This supports the suggestion that the tongue

might act as a reservoir for yeasts (Soames and Southam, 1985). For future work, the appropriate technique should be selected to best assess the objectives of the study. The oral rinse technique can be used where quantitative and overall carriage of yeasts are required; swabs can be used for examination of specific sites for carriage.

Previous studies of diabetics in which a mouth wash technique was used reported yeasts prevalence of 41% to 62% (Odds *et al.*, 1978; Tapper-Jones *et al.*, 1981; Fisher *et al.*, 1987; Lamey *et al.*, 1988; Hill *et al.*, 1989). In this study 66% of the diabetics were carriers of yeasts by the oral rinse technique.

The second objective was to compare species of yeasts isolated from patients with IDDM compared with those with NIDDM. *C. glabrata* and *C. tropicalis* were isolated more frequently from patients with either IDDM or NIDDM who wore dentures. There were significantly more carriers among individuals with IDDM compared with NIDDM subjects. No other study has found this association (Odds *et al.*, 1978; Tapper-Jones *et al.*, 1981; Lamey *et al.*, 1988). This might be due to the smaller sample size, differences in populations sampled and their distinction of IDDM from NIDDM based solely on treatment of the diabetic condition. In this study subjects were classified as IDDM or NIDDM according to family history of diabetes, clinical history of onset, requirement for insulin and progression of the disease.

By the API identification system, some studies have found 77% to 91% of their isolates to be *C. albicans* (Fisher *et al.*, 1987; Hill *et al.*, 1989; Darwazeh *et al.*, 1990). In this study only 54% of the isolates were *C. albicans* (Table 2.2); this figure includes the proportion that was not identified by API 20C Aux as *C. albicans* but found to be so by conventional methods (see Chapter 3 for more detailed discussion of methods for identification of yeasts). These results are similar to studies which found 60% of isolates from diabetics to be *C. albicans* (Odds *et al.*, 1978; Tapper-Jones *et al.*, 1981). This emphasises the need for accurate identification as patients with diabetes are more likely to carry and to have disease due to species other than *C. albicans* (Chapter 3) which might not be sensitive to routinely prescribed oral antifungal agents (Dick *et al.*, 1980).

The third objective was to assess the association between secretor status and carriage of yeasts among individuals with IDDM separately from those with NIDDM. In most diseases the influence of blood group or secretor status is marginal; sample sizes need to be large enough to discern such relationships with confidence (Mourant

et al., 1978). Because of the large number of patients in this study, those with IDDM could be analysed separately from those with NIDDM taking other factors into account by the use of multivariate discriminant analysis. By the univariate analysis, other studies of patients with diabetes found a relationship between non-secretion of blood group antigens and carriage of yeasts (Blackwell *et al.*, 1989a; Darwazeh *et al.*, 1990). These studies did not take into account the denture status (Blackwell *et al.*, 1989a) or type of diabetes (Darwazeh *et al.*, 1990) of its sample population. Another study did not report an association between secretor status and carriage of yeasts or development of disease (Lamey *et al.*, 1988); however, in this study, the number of subjects were much smaller, denture status and type of diabetes were not considered.

Among individuals with NIDDM, the results of the multivariate analysis show that non-secretion of blood group antigens was a marginally significant factor influencing carriage in the oral cavity as a whole. Palatal carriage of yeasts was not dependent on secretor status. Quantitative carriage of yeasts (assessed by the oral rinse technique) is associated with non-secretion; carriage *per se* does not depend on secretor status.

The fourth objective of the study was to dissect the contribution of various other factors to carriage of yeasts. The following factors were not associated with carriage by either univariate or multivariate analyses: gender; duration of diabetes; diabetic complications of neuropathy or nephropathy; alcohol consumption; contraceptive pill; systemic corticosteroid treatment; topical corticosteroid application; antibiotic usage; age, fit, occlusion or hygiene of denture; persistent albuminuria; or history of superficial *Candida* infections. Those for which significant associations were found are discussed below with reference to previous reports.

The prevalence of yeasts in the adult mouth has been shown to rise with age. The effects of age are not always easily separated from disease and medical treatment (Marples, 1960; Smits *et al.*, 1966). Age was not related to isolation of yeasts from diabetics (Tapper Jones *et al.*, 1981; Lamey *et al.*, 1988; Darwazeh *et al.*, 1990). In a Canadian study (Hill *et al.*, 1989) diabetics over 50 years of age had a higher density of yeasts compared with those less than 50 years old. Age was not isolated as a significant factor in the multivariate analysis, and the authors' suggested that carriage in the older age group might be due to the higher number of denture wearers. In this study, increase in age was associated with a decrease in the frequency of carriage, especially among non-denture wearers with IDDM (Tables 2.5 and 2.9). This was unexpected; one explanation might be that decreased isolation of yeasts with

increasing age reflects the efficiency of the mucosal immune defences against yeasts with increased frequency of challenge. Studies on vaginal carriage of yeasts found an inverse correlation between isolation of yeasts and levels of serum anti-*Candida* IgA (Schonheyder *et al.*, 1983). Secretory anti-*Candida* IgA has been reported to correlate with levels of serum IgA (Warnock and Hilton, 1976). Analysis of secretory and humoral immune responses of the study population to yeasts are needed to obtain evidence for this hypothesis.

Poor glycaemic control (HbA_1 and plasma glucose levels) was a significant factor associated with palatal carriage of yeasts among patients with IDDM who wore dentures. Carriage assessed by the oral rinse was not associated with HbA_1 levels; similar results were reported in other studies (Fisher *et al.*, 1987; Lamey *et al.*, 1988). The single study in which multivariate analysis was used found an association between HbA_1 level and colonisation determined by palate or denture base swab; however, this study did not differentiate individuals with IDDM from those with NIDDM (Hill *et al.*, 1989). Further evidence that diabetic control is associated with carriage was the significant associations between carriage and plasma glucose levels or persistent glycosuria among patients with NIDDM who did not wear dentures. Studies on other diabetic populations reported similar results (Odds *et al.*, 1979), but the majority did not find this association (Tapper-Jones *et al.*, 1981; Lamey *et al.*, 1989; Hill *et al.*, 1989; Darwazeh *et al.*, 1990).

Retinopathy was of marginal significance as a predictor of carriage among denture wearers with IDDM. This complication results from microvascular changes associated with diabetes which might also impair immune response through inadequate diffusion of tissue mediators and indicates that systemic changes associated with diabetes predispose to colonization.

Studies in non-diabetic populations reported a positive correlation between smoking and carriage of bacteria (Blackwell *et al.*, 1990; Blackwell *et al.*, 1992) and yeasts (Arendorf *et al.*, 1983). Among diabetics, smoking was a significant factor influencing carriage of yeasts; however, the type of diabetes and denture status were not taken into account (Tapper-Jones *et al.*, 1981). A significant association between carriage and smoking was found in this study; however, this association disappeared when results were analysed with respect to type of diabetes and denture status. This implies that these two factors were dependent variables in the overall univariate analysis.

In contrast to other studies which reported a higher prevalence and/or density of yeasts among diabetics who wore dentures (Tapper-Jones *et al.*, 1981; Lamey *et al.*, 1988, Fischer *et al.*, 1987; Hill *et al.*, 1989), we found no correlation with presence of denture and frequency or quantity of yeasts isolated with either technique. Similar results were reported by Darwazeh *et al.* (1990). There is no obvious explanation for these discrepancies. Continuous denture wearing might influence analysis of denture status results; however, we found this association only for palatal carriage among patients with NIDDM (Tables 2.8 and 2.9). Other studies found continuous presence of dentures is associated with increased frequency and density of yeasts (Tapper-Jones *et al.*, 1981; Darwazeh *et al.*, 1990).

The contribution of factors such as antibiotics and corticosteroids to carriage of yeasts is not easily differentiated from that of the underlying illness. Most of the studies in which associations between carriage and these treatments were found were carried out on hospitalized patients. The present study examined out-patients, the majority of whom were not on either of these treatments; therefore, no inferences can be made on the role of these therapies on oral carriage.

Chapter 3
Identification of yeasts

3.1 Introduction

The genus *Candida* contains more than 150 species. The common feature is the absence of any sexual form; but, they have a range of markedly different properties and they are classified in the genus by default rather than by design. The medically significant species represent only a minority subset of a large and widely disparate group.

Taxonomy is not a static discipline. Sexual forms of *Candida guillermondii*, *Candida kefyr* and *Candida krusei* have been described (Barnett *et al.*, 1983; Meyer *et al.*, 1984). The species *Candida paratropicalis* described in 1981 has not been accepted as distinct from *Candida tropicalis* by yeast taxonomists (Meyer *et al.*, 1984). In the cases of *Candida clausenii* and *Candida stellatoidea*, their synonymity with *C. albicans* has been acknowledged for a long time (Hasenclever *et al.*, 1961; Leth Bak and Stenderup, 1969; Lyon and Domer, 1985).

The most sweeping taxonomic revision was the merging of the two genera *Candida* and *Torulopsis*. Proponents of this fusion argue that separation on the basis of a single morphological character (production of pseudohyphae) is insufficient to warrant differentiation at the genus level. Against this are those who consider pseudomycelium formation as a valid and workable intergeneric distinction. Guanine:cytosine ratios of *Candida* species vary over a wide range which includes the *Torulopsis* species range of G:C ratio (Table 3.1). Endonuclease treatments of yeast DNA are at a preliminary stage but might provide more substantial evidence (Ashman *et al.*, 1990).

The principal unifying characteristics of the species presently classified in the genus *Candida* have been summed up by Meyer *et al.* (1984). The genus comprises yeasts with a variety of cell shapes, fermentation and assimilation reactions but without carotenoid pigments, ascospores, teliospores, ballistospores or arthrospores. Within the genus, species are separated primarily on the basis of their physiological properties. Each species has a specific pattern of abilities and inabilities to assimilate various organic compounds and to ferment particular carbohydrates. In some

Table 3.1 Estimates of moles % guanine and cytosine (% G + C) in DNA from medically important *Candida* species

Species	No. isolates tested	% G+ C
C. albicans	7	32.0 - 36.9
C. glabrata	6	35.5 - 40.2
C. guillermondii	>1	41.0 - 45.6
C. kefyr	>1	37.0 - 41.3
C. luscitaniae	>5	44.7 - 45.2
C. parapsilosis	>2	37.5 - 41.5
C. tropicalis	5	32.0 - 36.1
C. viswanathii	1	46.3

Table reproduced with modifications from Odds (1988)

instances, the patterns are very similar for two species and a close relationship can be inferred.

Morphological and physiological characteristics are by no means the sole criteria for the establishment of species. Serological tests, DNA base composition and DNA restriction fragment length polymorphism have shown differences between most of the pathogenic *Candida* species and confirms the traditional species separation (Meyer and Phaff, 1970; Tsuchiya *et al.*, 1974; Magee *et al.*, 1987; Mason *et al.*, 1987).

Strain differentiation of *C. albicans* has been pursued for obvious reasons. Methods examined include antigenic differences (serotype A and B) (Hasenclever *et al.*, 1961), cell dimension (Kockova-Kratochvilova *et al.*, 1963), susceptibility to lysis by virus (Mehta *et al.*, 1982), resistance to certain chemicals (Odds and Abbott, 1980), differences in enzyme activities (McReight and Warnock, 1982) and DNA restriction fragment length polymorphism (Scherer and Stevens, 1988). The latter offers distinction of the strains based on their genetic make-up compared with phenotypic differences the expression of which can be affected by the environment.

The most commonly encountered pathogenic *Candida* species usually grow well in aerobic cultures on rich or poor nutrient media (pH range 2.5-7.5) between 20-38 ^{0}C (Van Uden and Buckley, 1970). The optimum growth temperature of the two most virulent species, *C. albicans* and *C. tropicalis*, is closer to 37 ^{0}C (Lemos-Carolino and Madeira-Lopes, 1984). They all assimilate and ferment glucose as a carbon source; and, none use nitrate as a nitrogen source. They vary in their abilities to utilize other carbon and nitrogen sources (Meyer *et al.*, 1984). *Candida* species grow best under elevated concentrations of CO_2 in air; anaerobic conditions inhibit growth (Webster and Odds, 1987).

C. albicans is distinguished from other species by the following unique properties: formation of true mycelium; clamydospore formation (Beheshti *et al.*, 1975); and, germ tube formation in serum (Taschdjian *et al.*, 1960). All other *Candida* species form pseudomycelium; the members of the former genus *Torulopsis* do not form filaments. Very rare isolates of *C. tropicalis* are able to form clamydospores (Hasenclever, 1971); and, true hyphae and germ tubes have been reported to be formed by some *C. tropicalis* isolates (Martin and White, 1981; Joshi *et al.*, 1983). These "germ tubes" can be differentiated from *C. albicans* germ tubes by the presence of a septum or a marked constriction dividing the cell from the

filamentous portion (Hedden and Buck, 1980).

Clinically, identification of the causative yeast is not important. Isolation and demonstration of sensitivity to antifungal agents is sufficient for the care of a patient with fungal infection. Identification of yeasts results in accurate epidemiological accounts which are of local/international interest for recognition of novel yeast pathogens and shifts in sensitivity to antifungal agents.

Although detailed recommendations for the identification of *Candida* species from human sources vary, the general principles are the same. The yeast is isolated in a suitable primary culture, examined for pigmentation and the presence of *Cryptococcus neoformans*. The sample is then subjected to a set of tests for properties specific for *C. albicans*. If the isolate is not clearly identified as *C. albicans* on this basis, it is subjected to detailed tests for speciation (Milne, 1989).

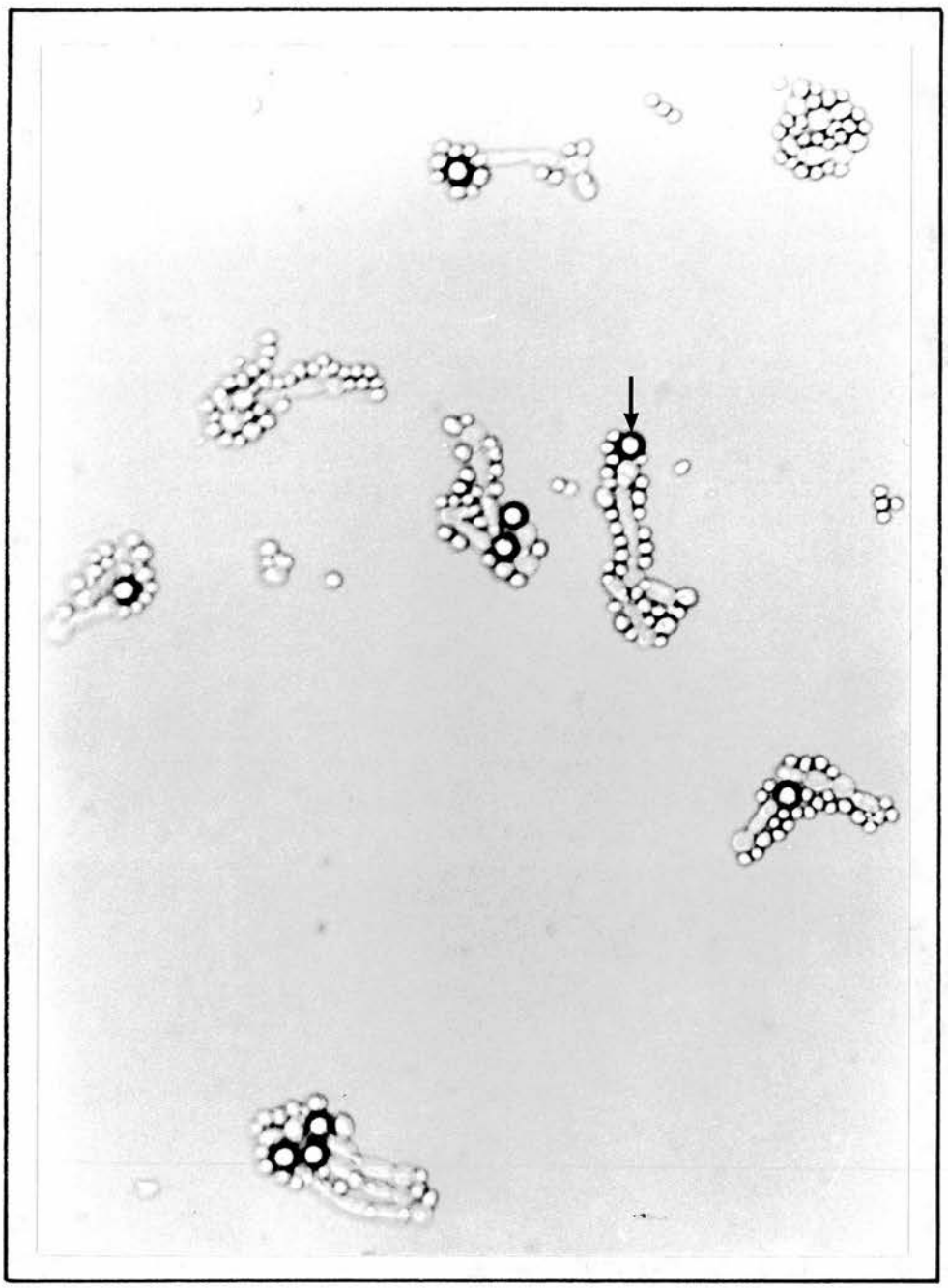
As the API 20C Auxanogram kit (API System S.A., France) has been used in several studies which have found higher proportions of *C. albicans* (77 - 91%) than in the present survey, the aim of this part of the study was to assess the accuracy of the kit in identification of *C. albicans* compared with conventional methods.

3.2 Materials and Methods

Details of the preparation of malt broths, cornmeal agar and Christensen's agar slopes are provided in the Appendix. These were obtained, ready for use, by the Media Preparation Laboratory, Department of Medical Microbiology, University of Edinburgh. The population examined and collection of samples has been described in Chapter 2; relevant information is repeated here. Five sites were swabbed: palate, floor of the mouth, tongue, right and left angles of the mouth. The swabs were placed immediately in malt broth and transported to the laboratory. Additionally, each subject rinsed his/her mouth with 10 ml of sterile PBS for 1 min and the contents were recovered. All samples were collected between 0900 and 1200 and processed within 1 hour.

The swabs in the malt broths were incubated at 37 ^{0}C for 24-36 hr before plating onto malt agar plates. The oral rinse was concentrated by centrifugation and resuspended in 1 ml of PBS; 20 ul of the suspension were plated onto a malt agar plate. The plates were incubated at 37 ^{0}C for 24-36 hr. Colonies were examined for

Plate 3.1: Production of filaments by *C. albicans* grown in cornmeal agar.



Magnification ca. 400. Arrow indicates a chlamydospore structure.

Table 3.2 Percent identification of species by the API 20C Auxanogram

Species	99.9-99.0%	98.9-96.6%	96.5-85.0%	< 85.0%	Total
C. albicans	506	22	18	0	546
C. glabrata	88	5	0	9	102
C. parapsilosis	12	0	20	0	32
C. cereviciae	21	8	3	0	32
*C. tropicalis**	10	4	0	8	22
T. beigelii	2	0	8	6	16
C. lusitaniae	1	5	0	2	8
C. guillermondii	4	0	4	0	8
C. pseudotropicalis	6	0	0	0	6
C. humicola	3	0	0	0	3
Rhodotorula spp.	0	0	1	2	3
C. lambica	0	0	2	0	2
C. laurentii	0	1	1	0	2
C. famata	1	0	0	0	1
Total					783

*includes one species classified as *C. parapsilosis at* 99.9%

Table 3.3 Isolates not identified to species level by the API 20C Aux.

Category	No.	Most frequent API profile*
Unacceptable	38	6743(3)7(3)
Doubtful	69	27(7)617(5)
Genus *Candida*	18	6000104
Low discrimination	13	6000004

*Other profiles differed in number occurring in parenthesis

Table 3.4 Germ tube positive isolates identified as other than *C. albicans* by the API 20C Auxanogram

Isolate	no	Most frequent API profile**	Differing sugar assimilation
Doubtful*	62	2(7)7617(5)	Ara (+)/ Mlz(+)
Unacceptable*	21	217213	Xyl(-)
*C. tropicalis**	19	25(5)6175	Mlz(+)
C. parapsilosis	10	6776175	Ara(+)/Mlz(+)
Genus *Candida*	8	6576175	Mlz(+)
C. lusitaniae	3	(6)472175	2Kg(-)/Mdg(-)/Mlz(+)
C. cerevuciae	2	2042007	2Kg(-)/Xyl(-)/Ado(-)/Mdg(-)/ Nag(-)/Tre(-)/Mlz(+)/Raf(+)
C. guillermondii	2	6776(3)77	Ara(+)/Mlz(+)/Raf(+)
C. humicola	1	6757776	Ara(+)/Ino(+)/Cel(+)/Lac(+)/ Raf(-)
T. beigelii	1	2556(7)77	Mlz(+)/Raf(-)
Total	129		

*At least one isolate with the stated profile was sent to the Mycology Laboratory, Western General Hospital, Edinburgh. Isolates were confirmed as *C. albicans*.

**Other profiles differed in number occurring in parenthesis.

C. albicans are negative for Raf, Lac, Cel, Ino; positive Glu, 2Kg, Gal, Mal

Glu= glucose, Gly= glycerol, 2Kg= 2-Keto-D-Gluconate, Ara= L-arabinose, Xyl= D-xylitol, Gal= galactose, Ino= inositol, Sor= sorbitol, Lac= lactose, Mdg= -methyl-D-glucoside Nag= N-acetyl-D-glucosamine, Cel= cellobiose, Mal= maltose, Sac= sucrose, Tre= trehalose, Mlz= melezitose, Raf= raffinose

morphology and pigmentation. Pure cultures were obtained by subculturing a single colony.

Cultures that appeared to be yeasts by Gram's stain were tested for germ tube production in horse serum after incubation for 2 hr at 37 ^{0}C. Care was taken to inoculate the horse serum lightly with the yeast.

Production of filaments by yeasts was tested using the Dalmau culture plate (Milne, 1989). Briefly, cornmeal agar plates were inoculated with yeasts and deep dilution strokes made by drawing the loop away from the well. A coverslip sterilized by flaming after dipping it in alcohol was placed on the plate so that it covered part of the tract and the well. The plates were incubated at 22 ^{0}C for 48 hr before examination. Plate 3.1 illustrates an isolate which produced filaments.

Urease production was determined only when sugar assimilation results and no filament production suggested that *Torulopsis* species might be present. A colony was inoculated into Christensen's agar slope and incubated at 30 ^{0}C for 24-48 hours; a colour change to red of the medium indicated urease production.

All API sugar assimilation strips were used as instructed by the manufacturer (API System S.A, France). The density of the inoculum was adjusted to 0.5 absorption units at 550 nm by a spectrophotometer (corresponds to approximately 2 x 10 ^{7} yeasts/ml) and cornmeal agar plates were used instead of ricemeal agar plates. The cupules were inoculated with the yeast in the API suspension medium, incubated at 30 ^{0}C in 5% CO_2 air mixture and read after 48 hours. A few strips were read between 48 and 72 hours and none required reading after 72 hours. The APILAB computer soft ware was used for identification of the isolate.

3.3 Results

Species identified correctly by the API 20C Aux system are shown in Table 3.2. *C. albicans* comprised 48.2%(546/1050) of all the isolates tested. The percent identification is a mathematical computation based on the results of each sugar assimilation test and indicates the probability of identity of the species. In order to display the results obtained in this study more clearly, the percent identification categories used in Table 3.2 are different from those used by the manufacturer. The manufacturer's categories are Excellent (99.9%), Very good (99.8-99.9%), Good

(98.9-88.9%) and Acceptable (88.8-85.1%). *C. albicans* was identified with >99.0% accuracy in most cases. *C. tropicalis* and *T. beigelii* were most often identified at <85% level; the manufacturer's level of "Unacceptable".

The categories of "Genus *Candida*" and "low discrimination" contained 18 and 13 isolates respectively. A further 69 isolates had a "doubtful" profile and 38 profiles were "unacceptable". The most frequent API profile in each of these categories is shown in Table 3.3.

A minority (129/1050, 12.3%) of germtube positive species were not identified as *C. albicans* by the kit. These included species that had been identified by the kit as *C. tropicalis*, *C. parapsilosis*, *S. cereviciae*, *C. guillermondi*, *C. luscitanea* and as doubtful/unacceptable profiles. The frequently encountered profile of these germ tube positive species is summarized in Table 3.4 with the variation in the sugar assimilation tests of the misidentified species. *Torulopsis* and *Rhodotorula* species were all correctly identified by the kit.

3.4 Discussion and Conclusions

C. albicans was frequently misidentified by the API 20C kit as *C. tropicalis* and *C. parapsilosis* confirming the similarities of these species. A large fraction of the isolates (107/1050, 10.2%) could not be identified to an acceptable level by the kit. Some could only be classed as "Genus *Candida*" or "low discrimination".

The majority of *C. albicans* isolates misidentified could assimilate melozoite; and, some strains could metabolise arabinose (Table 3.4). The API database from which the probable identification of species is computed is made up of clinical as well as reference isolates of yeasts. Only a small fraction of *C. albicans* included within the API database are able to utilize melozoite (4%) or arabinose (2%) (personal communication, API Laboratories, Basingstoke, U.K). The results indicate that for epidemiological studies as well as for clinical purposes, the germ tube test should be carried out for all yeast isolates. The manufacturer of the API 20C Aux. kit indicates the germtube test as an optional test. Conventional methods should be used to identify yeast species that are classed as "low discrimination" or "unacceptable" by the API computerbase. Milne (1989) has summarized the basic identification tests for yeasts to species level.

In conclusion, the lower proportion of *C. albicans* in this study compared with other studies among diabetic individuals is a characteristic of the population sampled. It was found that the kit under-represented *C. albicans* isolates; therefore, the prevalence figures of *C. albicans* quoted by studies which use the kit are probably higher after correcting for false-negative results obtained by this identification technique.

Chapter 4
Chronic Atrophic Oral Candidiasis

4.1 Introduction

Chronic atrophic oral candidiasis (denture stomatitis) is solely associated with wearing dentures. The studies of Cawson (1963) and Budtz-Jorgensen (1974) established the link between *Candida* and denture stomatitis. Subsequently, numerous studies have examined the prevalence of and factors predisposing to denture stomatitis. Presence and continuous wearing of dentures at night, dietary sucrose in the oral environment, traumatic effects of the denture, antibiotics and corticosteroid treatment, diabetes mellitus and smoking have all been implicated as potential factors contributing to development of denture stomatitis (see Section 1.3).

The genetic inability to secrete ABH blood group antigens in body fluids has been associated with a variety of infectious diseases (Blackwell, 1989). Non-diabetic individuals and pregnant women who are non-secretors are over-represented among patients with superficial *Candida* infections (Thom *et al.*, 1989). Non-secretors are also over-represented among carriers of *C. albicans* in normal subjects (Burford-Mason *et al.*, 1988) and patients with non-insulin dependent diabetes mellitus (Blackwell *et al.*, 1989a).

The aims of the study were:

1. to examine the prevalence of denture stomatitis and other oral conditions (angular cheilitis, fissured tongue, geographic tongue, median rhomboid glossitis, dry mouth and leukoplakia) among individuals with diabetes.

2. to examine the species of yeasts isolated from diabetic patients with denture stomatitis.

3. to assess the influence of secretor status on the development of denture stomatitis among patients with insulin-dependent diabetes (IDDM) and those with non-insulin dependent diabetes (NIDDM).

4. to assess the contributions of the following variables to the development of denture stomatitis: age; gender; duration of diabetic state; type of diabetes; control of

diabetes measured by glycosylated haemoglobin (HbA_1); diabetic complications - retinopathy, neuropathy and nephropathy; antibiotic usage; corticosteroid treatment; smoking; alcohol consumption; presence of dentures at night; type of denture; denture fit, extension, occlusion, age and hygiene; presence of persistent glycosuria and albuminuria; and history of superficial candidal infections.

4.2 Materials and Methods

The study population, epidemiological data obtained, isolation and characterization of yeasts have been described (Chapters 2 and 3). Similar statistical analyses were applied.

4.3 Results

Oral conditions among patients with dentures are compared with those without dentures in Table 4.1. Of the 216 denture wearers, 76 (35%) had denture stomatitis (Table 4.2). In 18% (14/76) the rinsing technique failed to isolate any yeasts. Similar results were obtained with the swab taken from the palate. The concordance between the results obtained by these methods was 78%. By MacNemar's test for paired alternatives, there was no significant difference between the discordant pairs obtained by the two sampling methods ($p > 0.1$). The species most frequently isolated from the palate of patients with denture stomatitis were *C. albicans* (33/76, 43%), followed by *C. glabrata* (6/76, 8%) and *C. tropicalis* (3/76, 4%). In 9%, the isolate could not be identified to a species level. Other species were isolated in 15% (11/76) of denture stomatitis cases (Table 4.3).

4.3.1 Univariate analysis

4.3.1.1 Blood group and secretor status

Denture stomatitis was more prevalent among individuals who are non-secretors than among secretors. This trend was not significant at $p \leq 0.05$. Analysis with respect to blood group and secretor status revealed that among individuals with blood group O with denture stomatitis, non-secretors (13/27, 48%) appeared to be more

Table 4.1 Prevalence of oral conditions among diabetic individuals

Condition	Prevalence among non-denture wearers (n = 223)		Prevalence among denture wearers (n = 216)	
Angular cheilitis	17	(8%)	25	(12%)
Denture stomatitis	0	(0%)	76	(35%)
Fissured tongue	10	(5%)	15	(7%)
Dry mouth	2	(1%)	13	(6%)
Geographic tongue	4	(2%)	7	(3%)
Median rhomboid glossitis	3	(1%)	7	(3%)
Hairy tongue	3	(1%)	5	(2%)
Glossitis	2	(1%)	5	(2%)
Leukoplakia	0	(0%)	2	(1%)
None	182	(82%)	61	(28%)

Table 4.2 Prevalence of denture stomatitis (DS)among patients with IDDM or NIDDM

Patient Category	With DS		Without DS		Total	
	no	%	no	%	no	%
IDDM	32	(41)	47	(59)	79	(100)
NIDDM	43	(32)	91	(68)	134	(100)
	75		138		213	

Missing = 3

$X^2 = 1.20$; $p = 0.27$

Table 4.3 Mycological profile of diabetic individuals (Palate swab)

Isolate	all denture wearers		with denture stomatitis	
	no.	(%)	no.	(%)
C. albicans	67	(31)	33	(43)
T. glabrata	13	(6)	6	(8)
T. beigelli	5	(2)	3	(4)
C. tropicalis	4	(2)	3	(4)
C. paratropicalis	3	(1)	2	(3)
S. cerevisiae	2	(1)	0	(0)
C. stellatoidea	1	($^1/_2$)	1	(1)
T. incospicua	2	(1)	1	(1)
C. humicola	1	($^1/_2$)	0	($^1/_2$)
C. pseudotropicalis	1	($^1/_2$)	1	(1)
Unidentified	24	(11)	7	(9)
Missing	5	(2)	2	(3)
Other	4	(2)	3	(4)
No isolate	83	(38)	14	(18)
Total	216	(100%)	76	(100%)

Table 4.4 Secretor status and prevalence of denture stomatitis (DS) among patients with diabetes

Patient category	+DS no.	(%)	-DS no.	(%)	χ^2	p
All						
Secretor	45	(31)	100	(69)	2.56	0.11
Non-secretor	24	(44)	30	(56)		
IDDM						
Secretor	18	(40)	27	(60)	0.03	0.87
Non-secretor	13	(45)	16	(55)		
NIDDM						
Secretor	27	(27)	72	(73)	1.90	0.17
Non-secretor	11	(44)	14	(56)		

p value refers to comparison of denture stomatitis between secretors and non-secretors

Table 4.5 Relationship between denture stomatitis (DS) and continuous denture wear

Patient category	continuous wear of denture	+DS no. (%)	-DS no. (%)	χ^2	p
Total*	Yes	45 (52)	42 (48)	23.2	<0.01
	No	7 (12)	53 (88)		
IDDM	Yes	18 (49)	19 (51)	3.50	0.06
	No	3 (18)	14 (82)		
NIDDM	Yes	27 (54)	23 (46)	18.82	<0.01
	No	4 (9)	39 (91)		

* Of a total of 216 denture wearers, 43 were recruited during the pilot study and were not questioned about their denture wearing habits. For 26 individuals this information was not recorded during the main study period.

Table 4.6 Multivariate analysis (Wilk's method)

Sample	% cases correctly predicted	Variables isolated
IDDM (n = 52)	63	glycosuria ($p < 0.05$)
NIDDM (n = 88)	82	Denture in at night ($p<0.01$) No. of yeast colonies ($p<0.01$) non-secretion ($p<0.01$)

prone to denture stomatitis than secretors (19/68, 28%); $\chi^2 = 2.69$, df = 1, p = 0.10. A similar trend was evident among individuals of blood group A; 45% (10/22) A non-secretors had denture stomatitis compared with 29% (15/50) A secretors ($\chi^2 = 1.1$, df = 1, p = 0.3). Secretor status of individuals of blood groups B and AB were not compared because the numbers were too small for statistical analyses.

4.3.1.2 Continuous wearing of denture

Significantly more individuals who did not remove their dentures at night (45) had denture stomatitis compared with those who removed their dentures at night (7) ($p < 0.01$). This was found particularly among individuals with NIDDM (Table 4.5).

4.3.1.3 Density of colonization

Subjects who did not have denture stomatitis had significantly fewer colony forming units (median = 10 cfu/ml) than subjects with denture stomatitis (median = 1850 cfu/ml, $p < 0.01$). This was seen particularly for patients with NIDDM (median = 1500 cfu/ml with denture stomatitis and 25 cfu/ml without denture stomatitis, $p < 0.01$). Those with IDDM did not show this relationship (median = 2400 cfu/ml with denture stomatitis, 502.5 cfu/ml without denture stomatitis, p = 0.07). Among subjects without denture stomatitis there was a non-significant trend of increased density of colonization of individuals with IDDM compared with those with NIDDM (p = 0.07).

4.3.1.4 Random plasma glucose

Lower values for random plasma glucose were observed among the 132 patients without denture stomatitis (median = 9.55 mmol/l) compared with those observed for the 72 patients with denture stomatitis (median = 10.95 mmol/l, Mann-Whitney p = 0.02). This was seen particularly among individuals with NIDDM (median = 10.75 mmol/l with denture stomatitis and 9.5 mmol/l without denture stomatitis, p = 0.04). Individuals with IDDM did not show this relationship (median 11.25 mmol/l with denture stomatitis and 10.15 mmol/l without denture stomatitis, p = 0.24).

4.3.1.5 Previous candidal infections

Among 33 individuals with a history of superficial candidiasis 18 (55%) had denture stomatitis compared with 58 (32%) of the 182 subjects with a negative history of candidiasis ($\chi^2 = 5.33$, df = 1, p = 0.02). Separate analysis with respect to insulin dependency revealed a trend for individuals with NIDDM with a history of candidiasis to be more prone to denture stomatitis: for IDDM individuals $\chi^2 = 1.2$, df = 1, p = 0.30; for individuals with NIDDM, $\chi^2 = 3.26$, df = 1, p = 0.07.

4.3.1.6 Variables not associated with denture stomatitis

No significant association was found between denture stomatitis and the following variables: gender; age; type of diabetes; control of diabetes (HbA_1); duration of diabetic state; ABO blood group; smoking; alcohol consumption; persistent glycosuria or albuminuria; denture fit, hygiene, occlusion, age, or type (partial or full); presence of diabetic complications (neuropathy, nephropathy, retinopathy); contraceptive pill; antibiotics; or corticosteroids (systemic or topical).

4.3.2 Multivariate analysis

In order to determine the relative contributions of variables to the development of denture stomatitis, a stepwise discriminant analysis (SPSSX) was used. Initially, all variables were screened at an F-value of 4 ($p < 0.05$). Analysis was then confined to the variables isolated as significant to increase the number of cases examined. Table 4.6 summarizes the contributory variables and the significance of their contribution in predicting infection. For patients with IDDM, the only variable identified by the analysis was persistent glycosuria ($p < 0.05$). For patients with NIDDM three factors were identified: presence of denture in the mouth at night ($p < 0.01$), number of yeasts isolated ($p < 0.01$) and non-secretion ($p < 0.01$).

4.4 Discussion

The results are discussed in the context of the objectives stated at the end of section 4.1. The first objective was to determine the prevalence of oral conditions among patients with diabetes. All forms of the oral conditions listed in Table 4.1 are

more prevalent among the patients with dentures than those without dentures. Fissured tongue was encountered in 6% of the diabetic individuals. This figure is similar to that reported for 2478 dental patients (Halperin *et al.*, 1953). The prevalence of geographic tongue and median rhomboid glossitis was slightly higher among diabetics than among dental and dermatology patients (McCarthy, 1941). The prevalence of all the above conditions were much lower than those reported for diabetic individuals by Farman (1976) who examined Coloured South Africans. These differences might be due to genetic and/or environmental factors between the predominantly North European population sampled here and that sampled by Farman. Dry mouth was a spontaneous complaint in only 3% of the subjects compared to 34% reported by Sharon *et al.* (1985). The univariate analysis showed that prevalence of denture stomatitis was similar among patients with IDDM or NIDDM. The prevalence of denture stomatitis reported (35.2%) here is within the range (24-60%) reported for non-diabetic individuals (Odds, 1988).

The second objective was to examine the species of yeasts isolated from diabetic patients with denture stomatitis. *C. albicans* was isolated from only 43% of the patients with denture stomatitis. Isolation of *C. glabrata* (8%), and other yeasts (28%) from patients with denture stomatitis indicate that, among diabetic individuals, species other than *C. albicans* are important causes of disease. The sensitivity of the oral rinse technique was comparable to that of the palate swab.

The third objective was to assess the contribution of secretor status to development of denture stomatitis. By univariate analysis, secretor status was not significantly associated with denture stomatitis among individuals with either NIDDM or IDDM. Multivariate analysis, however, revealed that non-secretors with NIDDM were more prone to development of denture stomatitis after adjusting for denture wearing habits and density of yeast colonization. This suggests that the univariate relationship might have been obscured by the other two contributory factors.

It has been suggested that patients with IDDM are more immunocompromised than those with NIDDM so that any protective effect of secretion of blood group antigens does not make a significant contribution to prevention of disease (Blackwell *et al.*, 1989a). A previous study did not find an association between secretor status and denture stomatitis among diabetic individuals (Lamey *et al.*, 1988); however, the number of patients examined was much smaller, there was no differentiation between IDDM and NIDDM, and multivariate analysis was not applied to the data.

The fourth objective of the study was to assess the contributions of other factors to development of denture stomatitis. The results are discussed in context of other published studies.

The following factors were not associated with denture stomatitis by either univariate or multivariate analyses: age; gender; duration of diabetic state; type of diabetes; control of diabetes (HbA_1); ABO blood group; smoking; alcohol consumption; persistent albuminuria; denture fit, hygiene, occlusion, age or type (partial or full); presence of diabetic complications (retinopathy, neuropathy, nephropathy); contraceptive pill; antibiotic usage; or treatment with corticosteroids.

In non-diabetic populations some studies found a significant association between denture stomatitis and the female gender (Davenport, 1970; Cawson, 1966), but one study did not (Budtz-Jorgensen *et al.*, 1975). Of the two studies which found an association between gender and denture stomatitis, one was a retrospective study; and, neither gave details of the proportion of the sexes in their sample population.

Corticosteroid inhalants have been reported in some studies to precipitate oral thrush, but other investigators have found no effect. Vogt (1979) concluded from an extensive literature review that use of inhalers was associated with 4-13% of oral candidal infections. In this study, all four patients on corticosteroid inhalers had no evidence of denture stomatitis. In the case of systemic corticosteroid treatment, 10/11(91%) of the recipients did not have denture stomatitis. There have been no reports on the effect of corticosteroids on denture stomatitis in man; but, it seems likely that the absence of denture stomatitis is due to the anti-inflammatory effects of corticosteroids.

The effect of antibiotics in precipitating superficial candidiasis is thought to be minimal (Walker *et al.*, 1979). In this study, antibiotics did not influence the development of denture stomatitis; however, the number of individuals on antibiotics was too few to draw firm conclusions.

Control of diabetes as measured by glycosylated haemoglobin (HbA_1) was not associated with denture stomatitis. Although this was unexpected, it is well established that blood glucose levels and salivary glucose levels among diabetic individuals do not correlate linearly (Sharon *et al.*, 1985).

Discriminant analysis indicated that factors contributing to development of denture stomatitis among patients with NIDDM are clearly different from those with

IDDM. Patients with NIDDM who do not remove their denture at night harbour a large number of yeasts; and, non-secretors of blood group antigens are particularly at risk of developing denture stomatitis. In contrast, none of these factors influence the development of denture stomatitis among individuals with IDDM. This is further supported by the results of the univariate analysis in which the p value for these factors among individuals with IDDM are consistently higher than the p value among NIDDM individuals.

Persistent glycosuria was the only predictor of denture stomatitis among patients with IDDM. The presence of 11.10 mmol/l or more of glucose in the arterial blood results in the appearance of glucose in the urine. It was unexpected that neither HbA_1 nor random plasma glucose were implicated as both are more precise indicators of glucose availability than glycosuria.

Identification of factors contributing to the development of denture stomatitis among individuals with IDDM and NIDDM might have implications for treatment of this condition. Treatment of denture stomatitis among patients with NIDDM might be similar to that in non-diabetic individuals i.e. removal of the denture especially at night (Turrell, 1966). Among patients with IDDM, treatment of denture stomatitis seems likely to depend on improvement of diabetic control reflected in persistent glycosuria.

Chapter 5
Adhesion of *Candida albicans* to buccal epithelial cells of secretors and non-secretors

5.1 Introduction

Colonization of the host is a pre-requisite for invasion by microorganisms. In the oral cavity, adhesion is an important factor contributing to colonization by microorganisms (Gibbons and Van Houte, 1975). The role of adhesion in pathogenesis of *Candida* was summarized in Section 1.4.1.1. Studies by different researchers on adhesion of *Candida* have reported conflicting results. Factors that influence *in vitro* studies of attachment of *Candida* to epithelial cells (which include the nature of the yeasts, the epithelial cells, the assay environment); methods of measuring attachment and mechanisms of adhesion will be outlined briefly.

5.1.1 Factors associated with yeasts

5.1.1.1 Concentration and viability

Both viable and non-viable yeasts adhere to epithelial cells. The ability of non-viable *C. albicans* to adhere was dependent on the severity of treatments used to kill the yeasts. Less rigorously treated yeasts retained their adherent capacities even though they were killed by these procedures; higher formalin concentrations or prolonged exposure to heat or ultraviolet light rendered *C. albicans* non-adherent (Lee and King, 1983). These results suggest that protein cell surface components might be involved in adherence. As yeast concentration increases so do the numbers that adhere to epithelial cells (King *et al.*, 1980), suggesting a "dose-dependent" pattern of binding with saturation of binding sites.

5.1.1.2 Phase and temperature of growth

The hyphal phase adheres better than blastospores by 2 to 50 fold (reviewed by Rotrosen *et al.*, 1986). Heat-killed germinated yeasts demonstrated no better adherence than heat-killed ungerminated yeasts and lower adherence than viable,

ungerminated yeasts. Factors which might contribute to the enhanced adhesion of hyphae include: qualitative changes in adhesin; expression of germtube-specific adhesins; and, increased surface area (Sobel *et al.*, 1981). The yeast phase represents the initial colonizing form and has been used often for *in vitro* experiments (Taschdjian and Kozinn, 1957). Compared with yeasts in the logarithmic phase of growth, adherence of organisms harvested in the stationary phase has been reported to be higher in one study and lower in another (King *et al.*, 1980; Segal *et al.*, 1982). Blastospores grown at 25-28 °C adhered to exfoliated vaginal epithelial cells (VEC) to a greater extent than did organisms grown at 37 °C (Lee and King, 1983; Segal *et al.*, 1982).

5.1.1.3 Growth medium composition

The adherence of some strains of *C. albicans* to buccal epithelial cells (BEC), VEC and acrylic is substantially increased after growth in defined media containing high concentrations of certain sugars (galactose, maltose or sucrose) as the sole carbon source (Douglas *et al.*, 1981; McCourtie and Douglas, 1984). Enhanced adherence appears to result from the production of an additional fibrillar-flocullar layer on yeast cell surfaces (McCourtie and Douglas, 1981). The induced changes in surface composition might be important *in vivo*, particularly in the mouth where high concentrations of sucrose and other dietary sugars are commonly found. Growth medium was the single most important factor influencing results of adhesion experiments (Kennedy and Sandin, 1988).

5.1.1.4 Species and strain

There is ample evidence for a hierarchy of relative virulence among the pathogenic *Candida* species (Odds, 1988). *C. albicans* is unquestionably the most virulent species, followed by *C. tropicalis* and *C. stellatoidea*. These differences were clearly reflected in the ability of the organisms to adhere to VEC and BEC (King *et al.*, 1980), vascular endothelium (Klotz *et al.*, 1983) and fibrin-platelet matrices (Maish and Calderone, 1980) *in vitro*. Differences in adhesion between strains of the same species are less obvious. Compared with isolates from active infections (I strains), isolates from asymptomatic carriers (C strains) were less virulent for mice (Segal *et al.*, 1984). Virulence for mice, adhesion and ability to synthesise the outer fibrillar-flocullar layer in response to high concentrations of

sugar were related to each other and were higher in I strains than C strains (McCourtie and Douglas, 1984). Adherence of isolates from oral thrush patients and those from healthy individuals were reported to be similar (Kearns *et al.*, 1983).

5.1.2 Factors associated with epithelial cells

5.1.2.1 Variability of surface components

Most studies of adhesion of *Candida* to mucosal epithelium rely on exfoliated cells. The resulting cell population is not uniform with respect to a number of properties: viability; contamination by commensal bacteria, yeasts, food debris; and, adsorbed immunoglobulins and secretory components (reviewed by Rotrosen *et al.*, 1986). Other factors such as age (Segal *et al.*, 1984) and phase of menstrual cycle (Botta, 1981) affect the adhesiveness of cells. Most researchers have used healthy comparable donors and extensive washing of cells in an attempt to minimize some of these factors. Despite such precautions, significant donor-to-donor variability, as well as day-to-day variability in studies restricted to a single donor, have been demonstrated (King *et al.*, 1980; Sobel and Obedeanu, 1983). The use of cell lines such as HeLa provide a homogeneous population free of contamination; however, cell surface characteristics might be altered and differ from those expressed by epithelial cells *in vivo*.

Adherence of yeasts to VEC has been reported to be greater than BEC (King *et al.*, 1980), less than BEC (Sobel *et al.*, 1981) or equal to BEC (Botta, 1981). *C. albicans* adhered to VEC of pregnant and diabetic women in greater numbers than to those from non-pregnant or non-diabetic women (Segal *et al.*, 1984). *C. albicans* adhered more to BEC from individuals with diabetes compared with non-diabetic controls (Darwazeh *et al.*, 1990). In neonates, receptivity of BEC to *C. albicans* is less at the time of birth than it is just a few days later (Cox, 1986); and, epithelial cells from infants who are colonized by *C. albicans* bind more yeasts than those from non-carriers (Cox, 1983).

5.1.3 The assay environment

5.1.3.1 Temperature, time and concentration of hydrogen ions

Optimum pH and temperature for *in vitro* adhesion are approximately 37 °C (King *et al.*, 1980) and pH 6-8 (Kimura and Pearsall, 1978; Cox, 1986). Although a 60 min incubation period was determined to be optimal for adhesion, results after 30 minutes incubation were not significantly different from those at 60 minutes (King *et al.*, 1980).

5.1.3.2 Bacteria

Influence of resident bacteria on colonization by *Candida* has been reported for many years. *Streptococcus salivarius* and *Streptococcus mitior*, but not *Streptococcus mutans*, reduced attachment of *Candida* to HeLa cell monolayer (Samaranayake and MacFarlane, 1982). Preattachment of lactobacilli diminished adhesion of *Candida* to VEC (Sobel *et al.*, 1981). Adherence of *Candida* to uroepithelial cells was enhanced if the cells were incubated with mannose-sensitive piliated Gram-negative rods (Centeno *et al.*, 1983); microscopic studies confirmed that the enhancement was a result of adhesion of *Candida* to the preattached bacteria. Strains of *Streptococcus viridans* coaggregate with *C. albicans in vitro* and might aid adherence indirectly to the oral epithelium; however, *S. mutans* and *S. salivarius* did not co-aggregate with *C. albicans* (Jenkinson *et al.*, 1990).

5.1.3.3 Antibodies

Antibodies to *Candida* might inhibit adhesion to BEC. There was an inverse relationship between titres of salivary IgA and adhesion of *Candida*; however, adhesion increased in 4 out of 13 experiments following partial removal of anti-*Candida* antibodies and in 4 of 20 experiments following removal of all antibody classes by immunoprecipitation (Epstein *et al.*, 1982).

5.1.4 Methods for measuring attachment

The principal methods of quantifying adhesion *in vitro* are light microscopy and radiometric assays. Light microscopy assay with yeasts was first developed by Kimura and Pearsall (1978). The basic method consists of mixing epithelial cells with yeasts and incubating for an appropriate period. Excess yeasts are removed by

centrifugation and the final mixture filtered through a membrane filter with a pore size of 8-12 um which theoretically retains epithelial cells and allows free yeasts to pass through. The filters are dried, stained and observed under a light microscope. The mean number of yeasts adherent to 100 epithelial cells and/or the mean percentage of epithelial cells binding yeasts is calculated. The radiometric assay utilizes yeasts labelled with radioiodine (King and Lee, 1980) or grown in nutrient tagged with C^{14} (Reinhart *et al.*, 1985). Scintillation counts of adherent yeasts are performed by immersing the filters in scintillation solution.

One of the technical problems in attachment studies is the tendency of yeasts to aggregate either alone or attached to epithelial cells making counting difficult or impossible. The light microscopy attachment assay is laborious, prone to subjective errors in counting and samples few epithelial cells. The radiometric assay is less laborious, less prone to subjective errors and rapid; the associated risks of handling radioactive materials is the main disadvantage of this technique. Both assays suffer from a common technical problem. Separation of host cells from non-adherent yeasts by filtration might generate a high reading of yeasts attachment to epithelial cells due to occlusion of the filter pores by epithelial cells. Particle trapping by filters might involve factors more complex than relative particle and pore size. In one study, 10-15% of an inoculum of *Candida* was retained presumably via electrostatic or hydrophobic forces by filters of the pore size routinely used in adherence studies (reviewed by Rotrosen *et al.*, 1986).

An automated image analysis for attachment of yeasts to *hard, transparent* surfaces was developed by Shakespeare and Verran (1988). The attachment was quantified by scanning for the presence of yeasts and calculation of the mean number of cells per unit area. This method is, however, not suitable for attachment studies using epithelial cells.

5.1.5 Mechanisms of adherence, adhesins and receptors

Adherence is defined here as a specific and essentially irreversible attachment of a microorganism to a host surface. Non-specific attachment involving van der Waal's forces, charge attraction, hydrophobic and surface tension might aid adherence through promoting the proximity of receptors and adhesins. The indiscriminate ability of *Candida* to attach to multiple surfaces has been suggested to indicate that no specific receptor molecules are necessary; adherence of yeasts to glass beads and

VEC was similar (Reinhart *et al.*, 1985). The interaction of yeasts with BEC is unlikely to be due to charge attraction since attachment was not substantially affected by pH value, high salt concentration or divalent cations (Lee and King, 1983). Hydrophobic forces influenced the attachment of *Candida* to dental resin materials; however, hydrophobicity did not correlate with adhesion implicating the influence of other mechanisms on adhesion (Minagi *et al.*, 1985). It has been suggested that hydrophobic forces might act indirectly to influence attachment to epithelial cells by promoting coaggregation of yeasts (Kennedy and Sandin, 1988).

Adherence of an organism to the host tissue commonly involves ligand-receptor interactions of the following three types (Ofek and Perry, 1985).

1. The ligand on the organism is a protein that specifically interacts with a carbohydrate receptor on the host cell, *e.g.* P fimbriæof *Escherichia coli* binding to Gal1-4Gal receptor on uroepithelial cells (Svanborg-Eden *et al.*, 1977).

2. The receptor on the host cell is a protein and specifically interacts with a carbohydrate on the microorganism. There is no conclusive evidence which indicates that proteins or peptides in the epithelial cell membrane are involved in the specific recognition of bacteria. It is, however, well accepted that carbohydrate structures on microorganisms bind to lectin receptors on macrophages (Freimer *et al.*, 1978).

3. Both the ligand and receptor are of the same nature i.e. polysaccharide or protein and bind via an intermediate substance such as Ca^{++} ions. The human complement-binding protein receptor which binds to C3d fragment is an example of such interaction (Ross *et al.*, 1985).

Numerous studies have been carried out to attempt to identify the adhesins of *C. albicans* which bind to host cells. Results of various investigators differ; but, there is compelling evidence that *Candida* mannoproteins can act as adhesins (reviewed by Rotrosen *et al.*, 1986). Pretreatment of *C. albicans* with a mannose-specific lectin, Concanavalin A, resulted in inhibition of attachment which could be restored by preincubation of the lectin with a specific haptenic sugar (D-methyl mannopyranoside) prior to the assay (Sandin *et al.*, 1982). Yeast adhesion was inhibited by preincubation of BEC with a crude mannoprotein preparation, indicating that the supernate contained the adhesin(s). The protein portion of this mannoprotein was more important than the carbohydrate moiety in mediating attachment to BEC (McCourtie and Douglas, 1985). Tunicamycin, an antibiotic which specifically inhibits the synthesis of mannoprotein in *S. cerevisiae*, resulted in a decreased

adhesion of *C. albicans* to BEC by over 60% compared to untreated organisms. It also inhibited the formation of the outer fibrillar flocullar layer (Douglas and McCourtie, 1983).

Mannoproteins are probably important in adhesion of *Candida* to cells other than BEC. Cell wall fragments of *C. albicans* pretreated with α-mannosidase did not adhere to VEC; blastospores pretreated in a similar fashion were unaffected (Lee and King, 1983). Adherence of *Candida* cell wall extract conjugated with sheep erythrocytes to fibrin-platelet matrix was abolished by pretreatment of the extract with α-mannosidase; however, there was no reduction of adherence of blastospores to fibrin-platelet matrices when they were pretreated in the same manner (Maish and Calderone, 1981). The null effect of α-mannosidase on *Candida* might be due to the inability of the enzyme to penetrate the intact cell wall (Lee and King, 1983).

One report proposed that chitin (a homopolymer of N- acetylglucosamine) which is localized to the inner layers of the cell wall of *Candida* might be involved in adhesion to VEC (Segal *et al.*, 1982). A soluble extract of chitin inhibited the adhesion of yeasts to human VEC and murine vaginal mucosa pretreated with the extract; fractionation revealed that the active component was low in protein and contained aminosugars (Lehrer *et al.*, 1988).

Most researchers view the evidence supporting an adhesive role for a deeply situated cell wall constituent as less compelling. Aminosugars are components of glycocalyx of host cell surfaces and candidal mannoprotein as well as chitin (Korn and Northcote, 1960). Mannose and N-acetylglucosamine share a common three dimensional structure except at the C-2 position; and, N-acetylglucosamine can compete with mannan for uptake by hepatic mannan-binding proteins (Kawasaki *et al.*, 1978). Additionally, more than one adhesin might be available on the surface of *Candida* (Critchley and Douglas, 1987b). This is not a novel concept; multiple adhesins have been shown to exist on *E. coli* (Svanborg-Eden *et al.*, 1977).

Participation of the protein portion of yeast mannoprotein in the adhesion process would be analogous to most bacterial adhesion mechanisms in which a proteinaceous adhesin interacts with a carbohydrate constituent of the host cell (Jones and Isaacson, 1983). It would also be consistent with the findings that yeast adherence to epithelial cells is not always inhibited by mannose (Sobel *et al.*, 1981), methyl α-D-mannosidase (Lee and King, 1983) or mannoprotein in which the protein component is absent or denatured (Lehrer *et al.*, 1983).

Fewer studies have investigated receptors for *Candida* on the host cell. The nature of BEC and VEC receptors appeared to be protein in some studies (Lee and King, 1983; Sobel *et al.*, 1981). Fibrinogen, a common mammalian cell surface glycoprotein, is a putative receptor for *Candida* (Skerl *et al.*, 1984). Other studies show glycosides such as L- and D-fucose, D-mannose and N-acetylglucosamine to be associated with receptors on buccal and vaginal epithelial cells (Sobel *et al.*, 1981; Critchley and Douglas, 1987a; Sandin *et al.*, 1982). The extracellular polymeric material (EP) from *C. albicans* 2346 could block attachment of the yeast to BEC by over 50%. Further studies of EP from five strains of *C. albicans* showed that EP from four of the strains primarily recognised L-fucose whilst one strain (*C. albicans* 2023) recognised N-acetyl D-glucosamine. L-fucose might be more commonly involved in adhesion of *C. albicans* (Critchley and Douglas, 1987b).

On the basis of epidemiological studies, a genetic characteristic, non-secretion of blood group antigens in body fluids, has been associated with susceptibility to some infectious diseases (Section 1.3.1.2). There was a significant inhibition of binding of two strains of *Candida* (3091 and 3118C) isolated from oral infections by pretreating the blastospores with boiled saliva from secretors whereas pretreatment of the blastospores with non-secretor saliva significantly enhanced attachment (Thom *et al.*, 1989). Binding of strain 3091 to non-secretor BEC pretreated with polyclonal anti-Le^a antisera was reduced; neither anti-Le^a nor anti-Le^b antisera inhibited binding of the two strains to secretor BEC (May *et al.*, 1989).

The objectives of this study were

1. to develop a flow cytometric assay for adhesion of yeasts to epithelial cells; and,

2. to examine the hypothesis that non-secretor cells bind more yeasts than secretor cells.

5.2 Materials and Methods

The composition of solutions and buffers are given in the Appendix.

5.2.1 Subjects

Donors were healthy individuals of blood group O who were matched for age and gender. Blood group was determined by slide agglutination with monoclonal anti-A and anti-B antibodies (Scottish National Blood Transfusion Service). Secretor status was determined by the haemagglutination inhibition assay using saliva (Mollison, 1983). The presence of Lewis antigens in the saliva of the donors was detected in an ELISA assay (Raza *et al.*, 1991) using monoclonal anti-Le^a and anti-Le^b antibodies (SAPU, Carluke, Scotland).

5.2.2 Collection of buccal epithelial cells (BEC)

BEC were collected between 0900h and 1100h by swabbing of the buccal mucosa with six cotton wool swabs per subject. The swabs were agitated in phosphate buffered saline (PBS) to release the cells. Cells were washed three times in PBS by centrifugation at 480g in a Sorvall RC2B centrifuge for 10 minutes each. Random checks on the cells revealed no yeasts attached to BEC on any occasions. The BEC were counted with a Neubauer Improved haemocytometer and resuspended in Dulbecco A + Supplement B to the desired concentration. Cells from each subject were analysed separately and were agitated vigorously to disrupt clumps prior to use in the adhesion assay.

5.2.3 Preparation of *C. albicans*

A freeze dried stock culture of *C. albicans* 2346 was reconstituted and subcultured onto malt agar and maintained by subculturing every other day. Once a month, a fresh freeze-dried stock culture was used. A loopful of *C. albicans* 2346 was inoculated into 100 mls of yeast nitrogen base (Difco) with 500 mM galactose and incubated for 24 hr at 37 °C with continuous shaking.

C. albicans was harvested after 24 hours and washed three times with distilled water by centrifugation at 480g for 10 minutes at each wash. The cells were counted with a Thoma counting chamber and suspended to a fixed concentration in PBS. Cells were in yeast form or budding phase; very few hyphae were present.

5.2.4 Labelling of *C. albicans*

Fluorescein isothyocyanate (FITC) (Sigma) was sonicated in 10mls of 1 M carbonate-bicarbonate buffer (pH 8.9) for 3 min at 1000 Hz. Yeasts were incubated in FITC for 30 min at 37 °C with continuous shaking. Labelling was also carried out by addition of 0.4 mg/ml of sonicated FITC to the growth medium and culturing at 37 °C overnight. Excess FITC was washed off with PBS by centrifugation at 480g for 10 min until the supernatant was clear. Rhodamine 123 (Sigma, U.K) was also examined as a fluorescent label; 200 ul of rhodamine (1mg/ml in PBS) was incubated with a pellet of yeasts for 10 minutes at 37 °C and the excess removed by washing the yeasts three times in PBS.

5.2.5 Adhesion assay

A suspension of BEC (0.2 ml of 2.5 x 10^5 BEC/ml) was mixed with 0.1 ml of *C. albicans* in a centrifuge tube (Elkay) and incubated with continuous shaking. The cells were washed three times by centrifugation at 18g for 5 min in 2 mls of PBS to remove unattached yeasts. This was the optimal centrifugation speed at which nearly all of the excess unattached yeasts were eliminated. Finally, the cells were fixed with 2% buffered paraformaldehyde before assessment by the flow cytometer (EPICS-C). The adherence of *C. albicans* to BEC was also examined in some experiments with the light microscope; a higher number of yeasts attached to BEC in the microscopic assay was roughly associated with a higher fluorescence value.

5.2.5.1 The flow cytometer - EPICS C

The EPICS "C" (Coulter Electronics, Luton, U.K.) equipped with a 5 W laser with a power output of 300 mW at 488nm was used to detect binding of fluorescein-labelled yeasts to BEC. The BEC were selected from the two parameter display of forward angle light scatter and 90° light scatter by a bitmap. The forward angle light scatter is proportional to the size of the particle and the 90° light scatter is proportional to the granularity of the particle. The background fluorescence was set at 1% by using the untreated BEC of the donor.

The amount of fluorescence associated with the BEC was an indication of the FITC-labelled yeasts adhering to the cell. Over 2000 BEC were analysed from each

subject. The readings were obtained at a photomultiplier sensitivity of 1000-1200 mV. The percentage (%) of BEC which were fluorescent above the background level was recorded on a one parameter histogram measuring fluorescence on a logarithmic scale. The mean fluorescent channel (MFC) value for the positive cells was obtained from a one parameter histogram measuring fluorescence on a linear scale.

The percentage of BEC which were read as positive when BEC were mixed with fluorescent yeast instantaneously was taken as the control reading.

5.2.6 Analysis of the results

The total fluorescence of the BEC of each sample was calculated by multiplying the mean fluorescent channel (MFC) value by the percentage of positive cells. This indicates the BEC population adhesiveness (PA). Statistical analysis was by multiple regression on logarithmically transformed data.

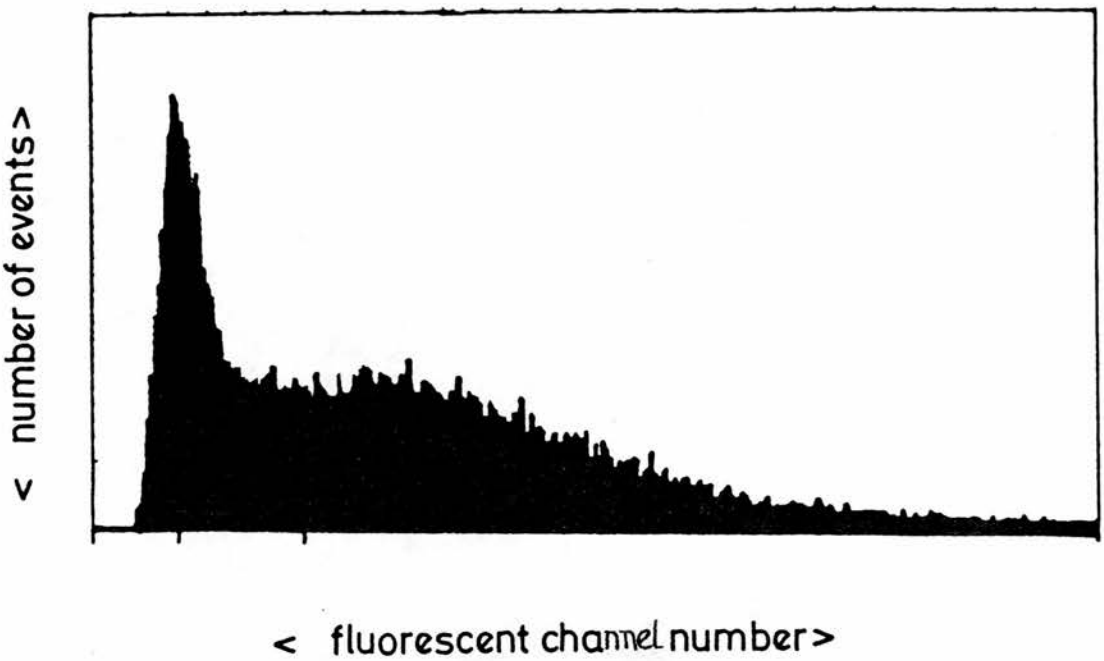
5.3 Results

5.3.1 Fluoresceination of yeasts

FITC binds to proteinaceous material (Rinderknecht, 1962). In order to achieve uniform fluoresceination of the yeasts, various methods were tried. *C. albicans* incubated overnight were brighter than those incubated for 30 min (% yeast fluorescent = 98%, mean fluorescent channel = 453; % yeast fluorescent = 97%, mean fluorescent channel = 259 respectively).

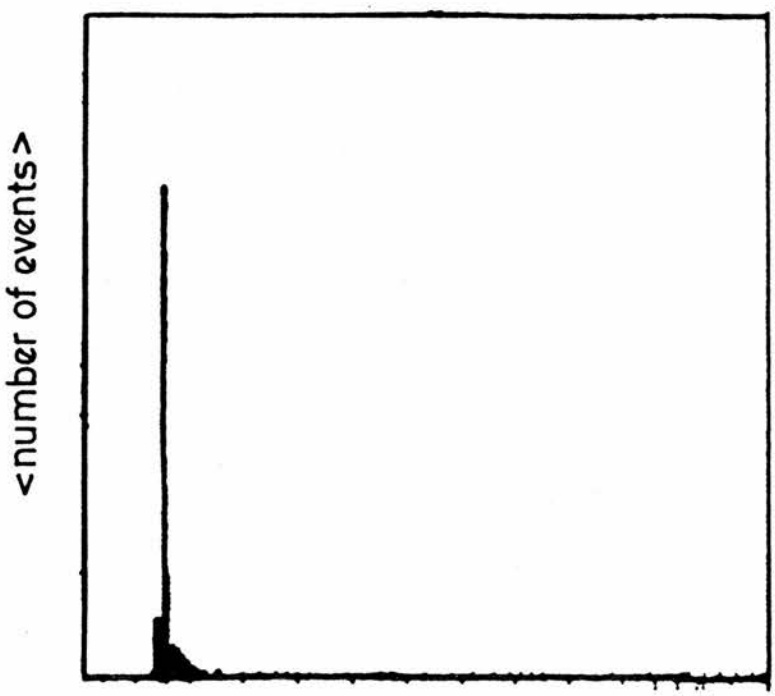
No difference in brightness was evident when yeasts were treated with 0.4mg/ml or 0.8 mg/ml of FITC. The standard deviation of fluorescence of FITC treated yeasts was high (Figure 5.1). Rhodamine which stains the mitochondria (Darzynkiewicz *et al.*, 1982) was examined; although the standard deviation of rhodamine-stained yeast was very low (Figure 5.2), these yeasts adhered to BEC at very reduced levels. Yeasts utilized in the adhesion assays were fluoresceinated for 30 min with 0.4 mg/ml FITC.

Fig 5·1 Fluorescence of FITC-treated C.albicans 2346



< fluorescent channel number >

Fig 5·2 Fluorescence of rhodomine-treated C.albicans 2346



<fluorescent channel number>

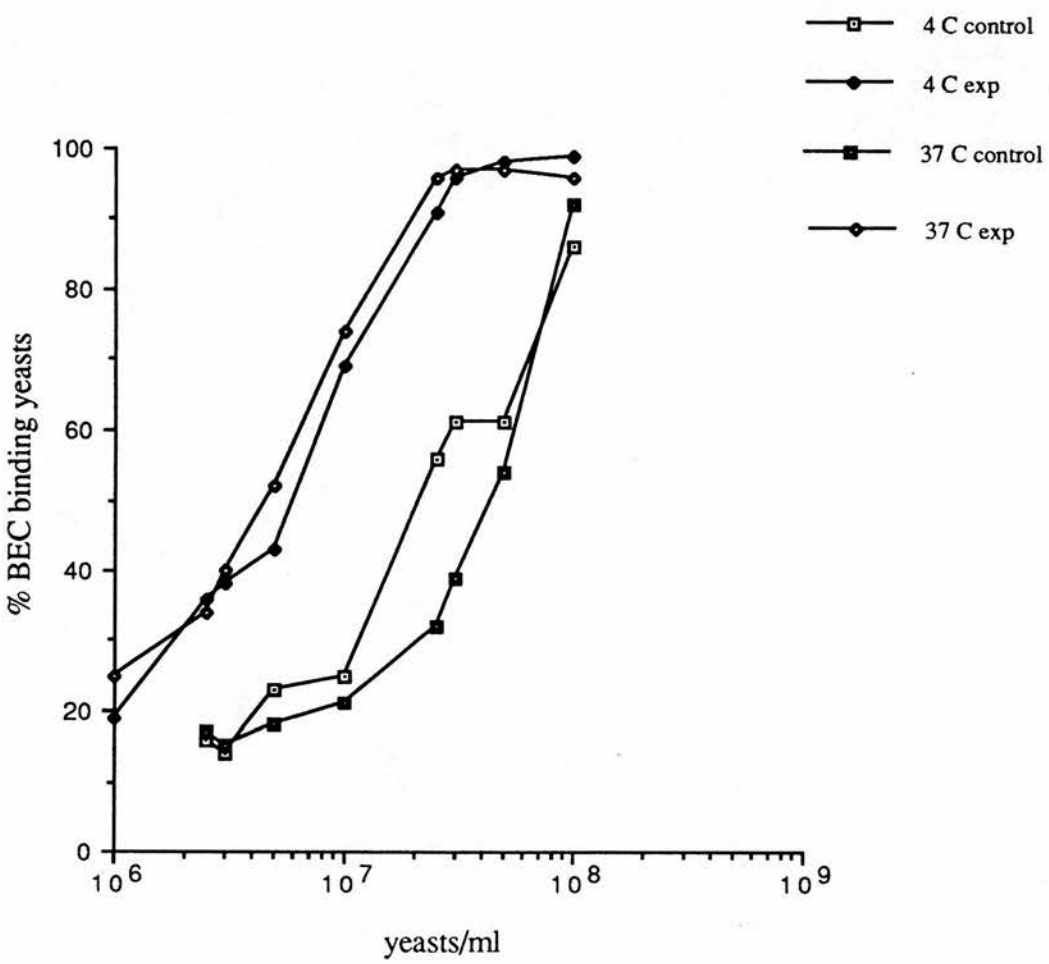


Figure 5.3: Effect of temperature and yeast concentration on the binding of *C. albicans* 2023 to 2.5 x 10^5/ml BEC

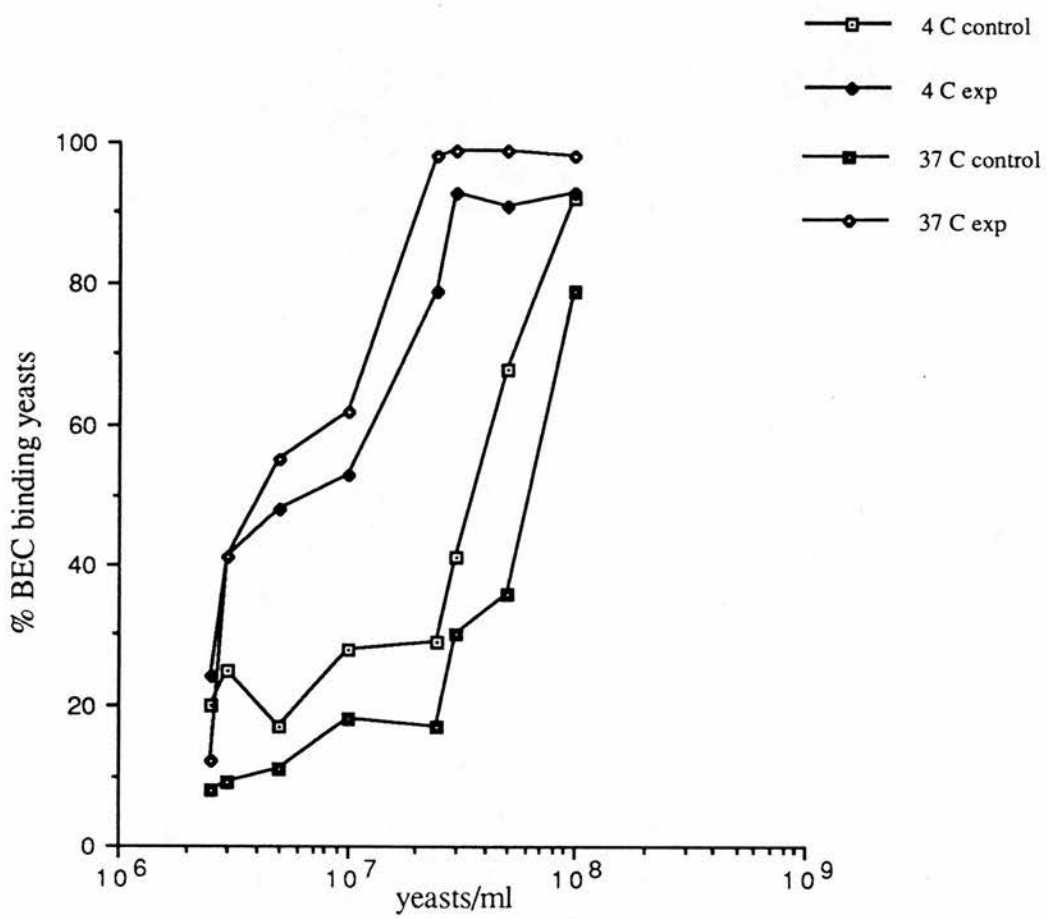


Figure 5.4: Effect of temperature and yeast concentration on the binding of *C. albicans* 2346 to 2.5 x 10^5/ml BEC

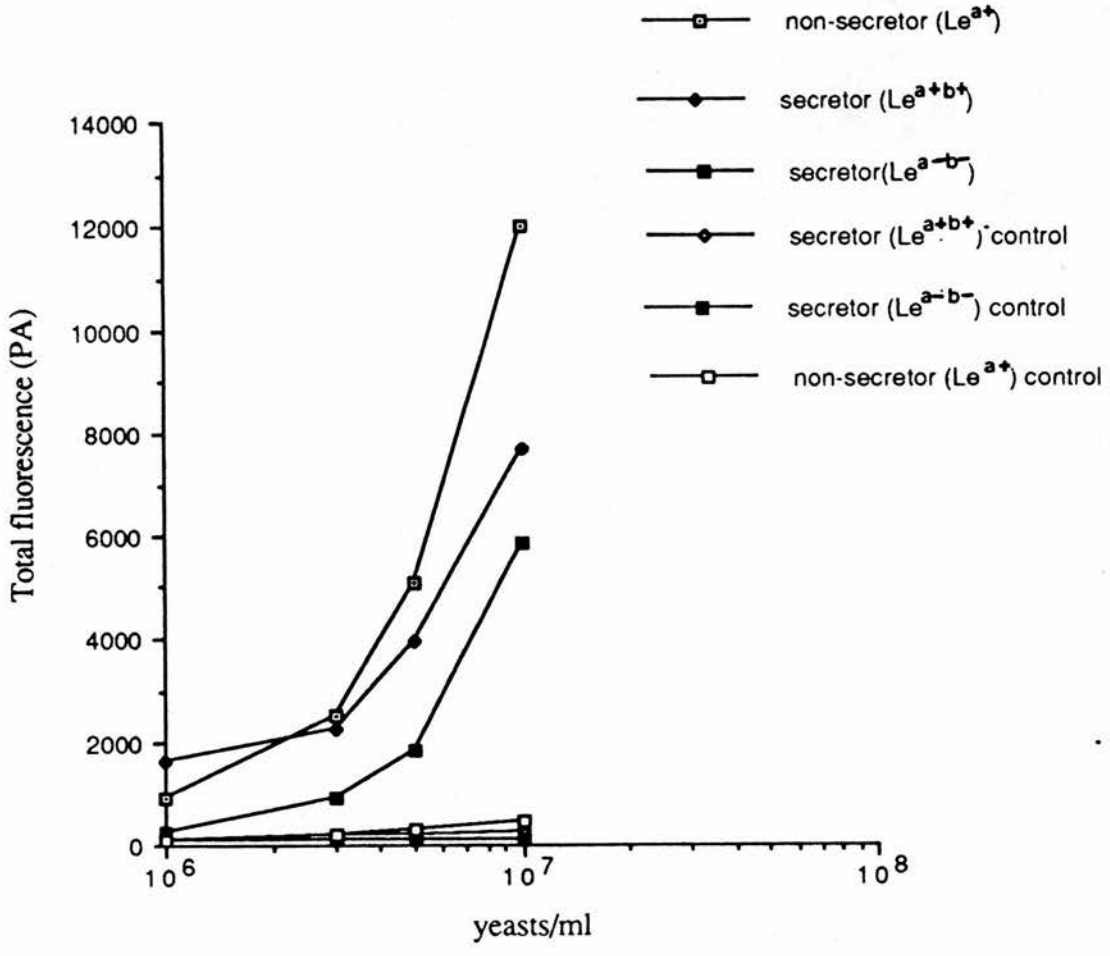


Figure 5.5: Binding of *C. albicans* 2346 to 2.5 x 10^5/ml BEC from non-secretors (Le^{a+}), secretors ($Le^{a+,b+}$) or secretors ($Le^{a-,b-}$)

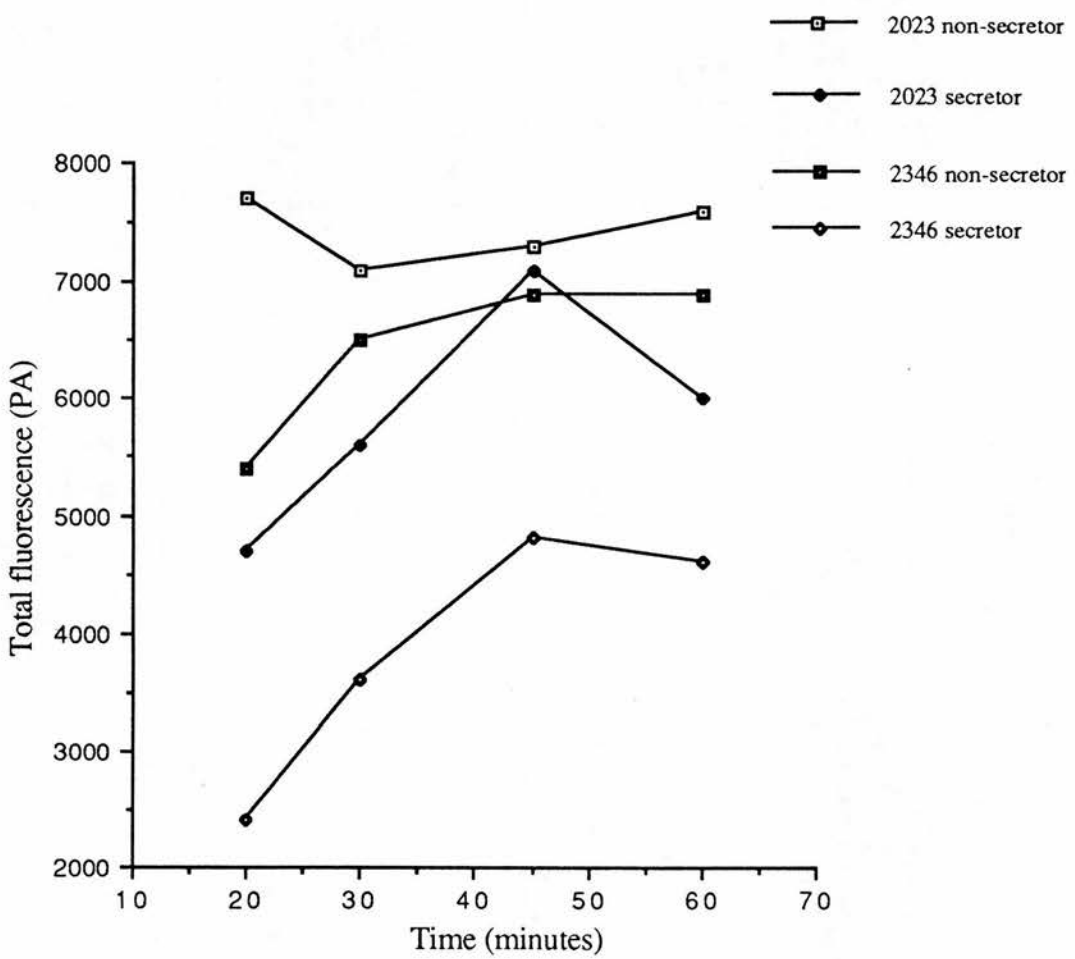


Figure 5.6: Time course of attachment of *C. albicans* to 2.5 x 10^5/ml BEC expressed as total fluorescence (PA)

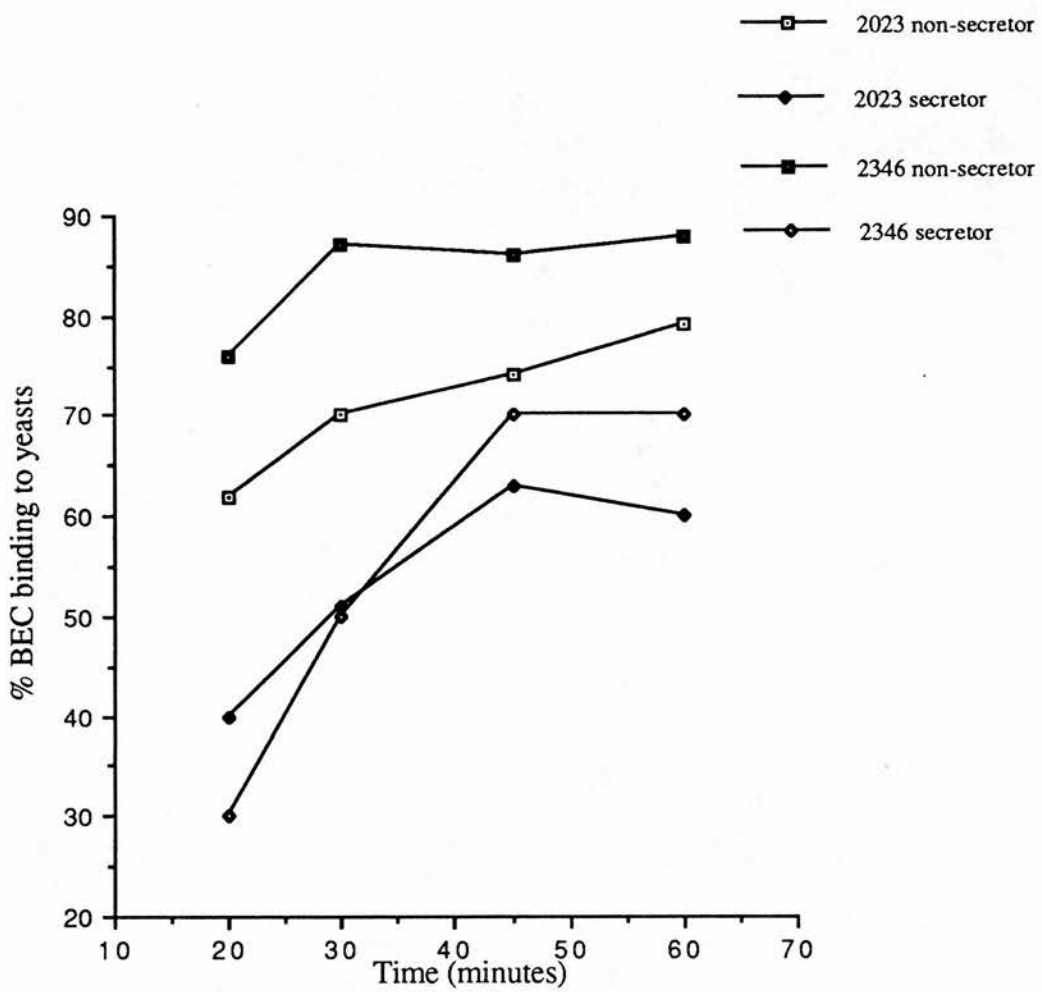


Figure 5.7: Time course of attachment of *C. albicans* to 2.5 x 10^5/ml BEC expressed as % BEC binding yeasts

Table 5.1 Adherence of *C. albicans* 2346 to blood group O BEC

Experiment	Secretor status*	% BEC	MFC	PA (10^3)
1	secretor	76	101	7.7
	non-secretor	84	143	12.0
2	secretor	96	306	29.4
	secretor	97	544	52.8
	secretor	92	258	23.7
	non-secretor	98	346	34.9
	non-secretor	97	517	50.2
	non-secretor	96	695	66.7
3	secretor	93	222	20.6
	secretor	94	202	19.0
	secretor	62	117	7.3
	secretor	84	204	17.1
	non-secretor	93	228	21.2
	non-secretor	84	496	41.7
	non-secretor	62	186	11.5
	non-secretor	92	297	27.3

MFC = Mean linear Fluorescent Channel
PA = Population Adherence (MFC x %BEC)
*All donors expressed the Lewis antigen.
Multiple regression analysis of difference between secretors and non-secretor BEC binding to yeasts $p = 0.07$

Table 5.2 Adherence of *C. albicans* 2023 to blood group O BEC

Experiment	Secretor status*	% BEC	MFC	PA(10^3)
1	secretor	56	132	7.4
	secretor	72	140	10.1
	secretor	86	101	8.7
	secretor	75	90	6.8
	non-secretor	80	107	8.6
	non-secretor	97	120	11.6
	non-secretor	70	132	9.2
	non-secretor	68	150	10.2
2	secretor	90	99	8.9
	secretor	67	104	7.0
	secretor	54	103	5.6
	secretor	86	109	9.4
	non-secretor	54	102	5.5
	non-secretor	97	105	10.2
	non-secretor	69	87	6.0
	non-secretor	73	94	6.9

MFC = Mean linear Fluorescent Channel
PA = population adherence (MFC x %BEC)
*All donors expressed the Lewis antigen
Multiple regression analysis of difference between secretors and non-secretors BEC binding to yeasts p = 0.65

Table 5.3 Comparison of adhesion of *C. albicans* strains 2023 and 2346 to blood group O BEC

Experiment	*C. albicans* strain	Secretor status*	% BEC	MFC	PA (10^3)
1	2346	secretor	80	205	16.5
	2023	secretor	55	213	11.8
	2346	non-secretor	87	202	17.5
	2023	non-secretor	71	151	10.8
2	2346	secretor	56	315	17.7
	2023	secretor	48	146	7.0
	2346	non-secretor	70	171	12.0
	2023	non-secretor	55	205	11.2
3	2346	secretor	70	100	7.0
	2023	secretor	78	150	11.7
	2346	non-secretor	50	599	30.0
	2023	non-secretor	70	348	24.0

MFC = Mean linear Fluorescent Channel
PA = Population Adherence (MFC x %BEC)
*All donors expressed the Lewis antigen
Multiple regression analysis of differences between C. albicans 2023 and 2346 $p > 0.05$.

5.3.2 *C. albicans* concentration

The optimal concentration of *C. albicans* 2023 and 2346 used in the binding assay was determined experimentally (Figure 5.3 and 5.4). Control values represent readings obtained when BEC and yeasts were mixed instantaneously and read by the flow cytometer. These values were low at yeast concentration of 10^7 ml^{-1}. Higher control values represent yeasts and BEC traversing the path of the detecting laser stream at the same time and being read as adherent yeasts. At a concentration of 10^7 ml^{-1} of yeasts, corresponding to BEC:yeast ratio of 1:40, the epithelial cell receptors were not fully saturated and the background control values were low. Using BEC from donors who were $Le^{a+,b+}$ (secretors), $Le^{a+,b-}$ (non-secretors) and $Le^{a-,b-}$ (secretors), maximum differences were similarly found at yeast concentration of 10^7 ml^{-1} (Figure 5.5).

5.3.3 Period and temperature of incubations

Attachment of yeasts to BEC after 45 min incubation was selected because it represented a period where binding was complete (Figure 5.6 and 5.7). There was no difference in attachment at 4 °C compared with that at 37 °C (Figure 5.3 and 5.4); an incubation temperature of 37 °C was chosen because it approximates physiological conditions. The background control values were low at yeast concentration of 10^7 ml^{-1}. These parameters were similar for both strains.

5.3.4 Attachment of *C. albicans* to secretor and non-secretor BEC

C. albicans 2346 attached preferentially to non-secretor BEC compared to secretor BEC; however, this difference was not significant at $p < 0.05$ (Table 5.1). Experiments with *C. albicans* 2023 also showed that non-secretor BEC bound more yeasts compared with secretor BEC (Table 5.2). *C. albicans* 2023 was not significantly different from *C. albicans* 2346 (Table 5.3).

5.4 Discussion

The results are discussed in the context of the objectives stated at the end of Section 5.2. The majority of studies of adherence of yeasts to exfoliated epithelial cells *in vitro* have been carried out by light microscopy and are severely limited by technical considerations already discussed. These limitations have been overcome in this study by the use of fluoresceinated *C. albicans* and analysis of the adherence by flow cytometry. The advantages of flow cytometry are: over a thousand BEC can be analysed within a few minutes; it is less prone to subjective errors; and, unattached yeast cells are not recorded by the instrument.

The variation in fluoresceination of yeasts was probably due to budding cells which were at different growth and maturity phases. Although this variation was reduced by using rhodamine, these yeasts did not adhere to BEC for reasons that are not obvious. The binding of rhodamine to *C. albicans* mitochondria might suppress respiration and reduce viability of yeasts.

An assay temperature of 37 °C was used to mimic as closely as possible the physiological conditions although no difference in attachment was observed at 4 °C compared with 37 °C. Experiments on the effect of pH on adhesion were not necessary because pH in the mouth varies widely and, other studies have shown no influence of pH on binding of yeasts to BEC (section 5.1.3.1).

The second objective was to examine the difference in binding of yeasts to BEC from secretors and non-secretors. *C. albicans* 2346, which has fucose-recognising adhesin, bound in greater numbers to BEC from non-secretors compared with those from secretors; the 95% confidence limit of binding of yeasts to non-secretors BEC was 91% to 720% more compared with secretors BEC ($p = 0.07$). There was no difference in the binding of *C. albicans* 2023 to BEC from secretors or non-secretors ($p = 0.65$; 95% confidence limits 65% to 194%). The major adhesin on *C. albicans* 2023 binds N-acetyl D-glucosamine which might account for these results. There was no difference in the binding of *C. albicans* 2023 and 2346 to BEC. This is compatible with the fact that both isolates are from active infections.

A recently published study (Tosh and Douglas, 1991) reported that *C. albicans* 2346 bound in significantly greater numbers to BEC from non-secretors compared with those from secretors; however, the number of donors was small (numbers not

stated).

If the fucose determinant of H were the receptor, BEC of secretors would have bound more yeasts than non-secretors BEC because secretors have significantly greater amounts of H in the form of H Type 1 on their epithelial cells (Rahat *et al.*, 1990). Similarly, Le^b which is present exclusively on BEC from secretors, cannot be the receptor for the fucose-recognising adhesin on *C. albicans* 2346 that accounts for the increased binding of yeasts to BEC from non-secretors. The Le^a determinant might be one of the receptors for the fucose-recognizing adhesin of *C. albicans* 2346 because it is usually found in greater quantities on BEC from non-secretors and non-secretors bound more yeasts than secretors. Individuals who were secretors but did not express the Lewis antigens (*le/le*) bound fewer yeasts than either secretors or non-secretors who expressed the Lewis antigens (Figure 5.5).

Although secretors express Le^b predominantly, some of these individuals have substantial amounts of Le^a in their body fluids (Ogata *et al.*, 1988). Monoclonal anti-$Lewis^a$ has been shown to bind to cells of secretors; but the amount detected varied greatly compared with uniformly high levels of binding observed for BEC from non-secretor (Blackwell *et al.*, 1992, in press).

In conclusion, the fucose molecule present in the H Type 1 and Le^b determinants might bind to the adhesin on *C. albicans* 2346 but do not account for the increased binding of yeasts to non-secretor cells. The role of Le^a determinant as a receptor is explored in the next chapter.

Chapter 6
The role of blood group antigens as epithelial cell receptors for *Candida albicans*

6.1 Introduction

In the context of the qualitative and quantitative distributions of H Type 2, H Type 1 and the Lewis antigens on cells of secretors and non-secretors, the results of the previous chapter suggest that Le^a might be a receptor for the fucose-recognising adhesin of strain 2346.

The cell wall of *C. albicans* has been studied by extraction techniques, conjugated lectins and antibodies and chemical stains. Figure 6.1 depicts the layers and components of the cell wall of *C. albicans* (Odds, 1988). There is general agreement that mannan polysaccharides are located throughout areas of high electron density and that the innermost layer is rich in chitin and β-1,6 glucans (Evron and Drewe, 1984; Poulain *et al.*, 1985a). Glucans are probably the major components of the electron transparent layer adjacent to the innermost layer (Poulain *et al.*, 1985a). Lipids are located in several wall layers (Poulain *et al.*, 1985b).

The outer fibrillar layer, also known as the extracellular polymeric material (EP), is not peculiar to *in vitro* grown cells. A number of investigators have described a prominent fibrillar-floccular layer on *C. albicans* in scrapings taken from the tongue and buccal mucosa of patients with oral candidiasis (Howlett and Squier, 1980; Marrie and Costerton, 1981). Its synthesis is depressed by tunicamycin, an inhibitor of mannoprotein synthesis in *S. cerevisiae* (Douglas and McCourtie, 1983). The lectin Concanavalin A can agglutinate *C. albicans*, confirming that there is mannan at the outer surface of the cell wall (Janson and Paktor, 1977; Cassone *et al.*, 1978). This layer can be easily removed by mild procedures such as treatment with hot salt solutions or even water washes (Sikl *et al.*, 1964; Evron and Drewe, 1984). This loosely bound material can be found in *C. albicans* culture filtrates (Massler *et al.*, 1966), although it is unclear whether mannoproteins from culture filtrates have been actively exported by the cells, leached into solutions or released by autolysis.

Electron microscope studies suggest that EP is involved in adhesion of *C. albicans* to buccal and vaginal epithelial cells (Marrie and Costerton, 1981;

Calderone *et al.*, 1984). This interaction has been described as a loose attachment *in vivo*; but, *in vitro* the yeasts appear to lie close to or within the epithelial cells (Calderone *et al.*, 1984). Other studies have shown that EP contains yeast adhesin(s) and that the protein portion of the mannoprotein is more important in mediating adhesion to epithelial cells. Chemically it is composed of 65-82% carbohydrate (mannose is the main component), 7% protein, 0.5% phosphorous and 1.5% glucosamine (McCourtie and Douglas, 1985).

The objective of this part of the study was to assess the ability of the EP from *C. albicans* 2346 to bind purified glycoconjugates carrying blood group determinants.

6.2 Materials and Methods

The compositions of buffers and solutions are given in the Appendix.

6.2.1 Growth of *C. albicans*

A loopful of *C. albicans* 2346 grown on malt agar was used to seed the yeast nitrogen base (Difco) containing 500 mM galactose medium. Yeasts were incubated for 24 hr at 37 °C with shaking.

6.2.2 Extraction of EP

EP was obtained by the method of Critchley and Douglas (1987a). Batches of medium (500 ml in 2 litre Erlenmeyer flasks) were inoculated with overnight yeast cultures (50 mls) and incubated at 37 °C for 5 days in an orbital shaker. Yeasts were removed by centrifugation and the culture supernatant (500 ml) was dialysed for one week against 5 changes (12 litres) of distilled water. The retentate (EP) was freeze dried, weighed and stored at -20 °C. The EP was dissolved in PBS before use and the protein concentration was determined by the Coomassie brilliant blue assay (Bradford, 1976).

6.2.3 Adhesion assay

Buccal epithelial cells (0.2 ml of 2.5 x 10^5 cells/ml) were incubated for 30 min at 37 °C with 0.2 ml of EP. Excess EP was removed by washing three times with PBS and resuspending the BEC to the original concentration in Dulbecco's PBS with supplement B. Control BEC were incubated with PBS only. The ability of EP-treated and PBS-treated BEC to bind fluorescein-labelled yeasts was examined by flow cytometry as described in Chapter 5.

6.2.4 ELISA assay for the detection of binding of EP to blood group antigens conjugated to human serum albumin (HSA)

The glycoconjugates used are illustrated in Figure 6.2. They are manufactured by BioCarb (Lund, Sweden) and were obtained from Russel Fine Chemicals, Chester, U.K. The method described by Gray (1979) was used to attach EP from strain 2346 to microtitre plates. EP (0.2 ml of 1mg/ml) was added to 1 ml of NaOH (0.01N) containing a phenolphthalein indicator. When the solution turned pink, 1 mg of cyanuric chloride was added. After 10 s the mixture was added to 0.1 ml poly-L-lysine (0.1%) and incubated for 1 hr at 4 °C. The conjugated EP (100 ul) was pipetted into polystyrene microtitre wells (M129B, Dynatech, Sussex, U.K) and left at room temperature for 24 hr with constant shaking. The wells were washed three times with distilled water; 100 ul of distilled water was added to each well and the plates stored at 4 °C.

Each well was washed with distilled water before blocking with blocking buffer for 1 hr. Unless otherwise noted, all procedures were carried out at room temperature. The plates were washed three times with washing buffer and 40 ul of one of the glycoconjugates (1 mg/ml in PBS) diluted with 10 ul of blocking buffer was added and incubated for 1 hr. The plates were washed and biotinylated chicken anti-HSA (Sera-Lab LTD, Sussex, U.K) (100 ul/well, dilution 1/100 in blocking buffer) was added and left for 1 hr. Unbound antibody was removed by washing three times and streptavidin biotinylated HRP complex (Amersham, U.K) (100 ul, dilution 1/100 in blocking buffer) was added. The plates were washed three times and 100 ul of substrate solution, 40 mg O-phenylenediamine in 100 ml 0.1 M citrate buffer activated immediately before use with 40 ul of 30% (v/v) H_2O_2, was added and the reaction allowed to develop in the dark for 10 to 15 min. The reaction was stopped by adding 50 ul of 12.5% H_2SO_4. The colour development was read by a

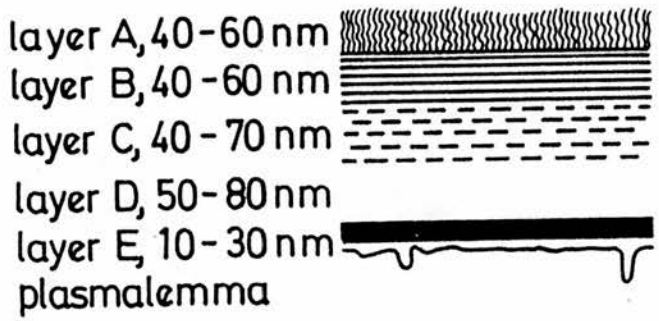


Figure 6.1 Diagrammatic representation of the cross-sectional structure of a *C. albicans* blastospore cell wall. The overall wall thickness is approximately 250-260 nm. Layer A: electron-dense fibrils, mostly mannoproteins; Layer B: electron-dense, mostly mannan; Layer C: weakly electron-dense, may contain mannan and glucan; Layer D: electron-transparent, mostly glucan; Layer E: strongly electron-dense, chitin and glucan with some mannan (Odds, 1988)

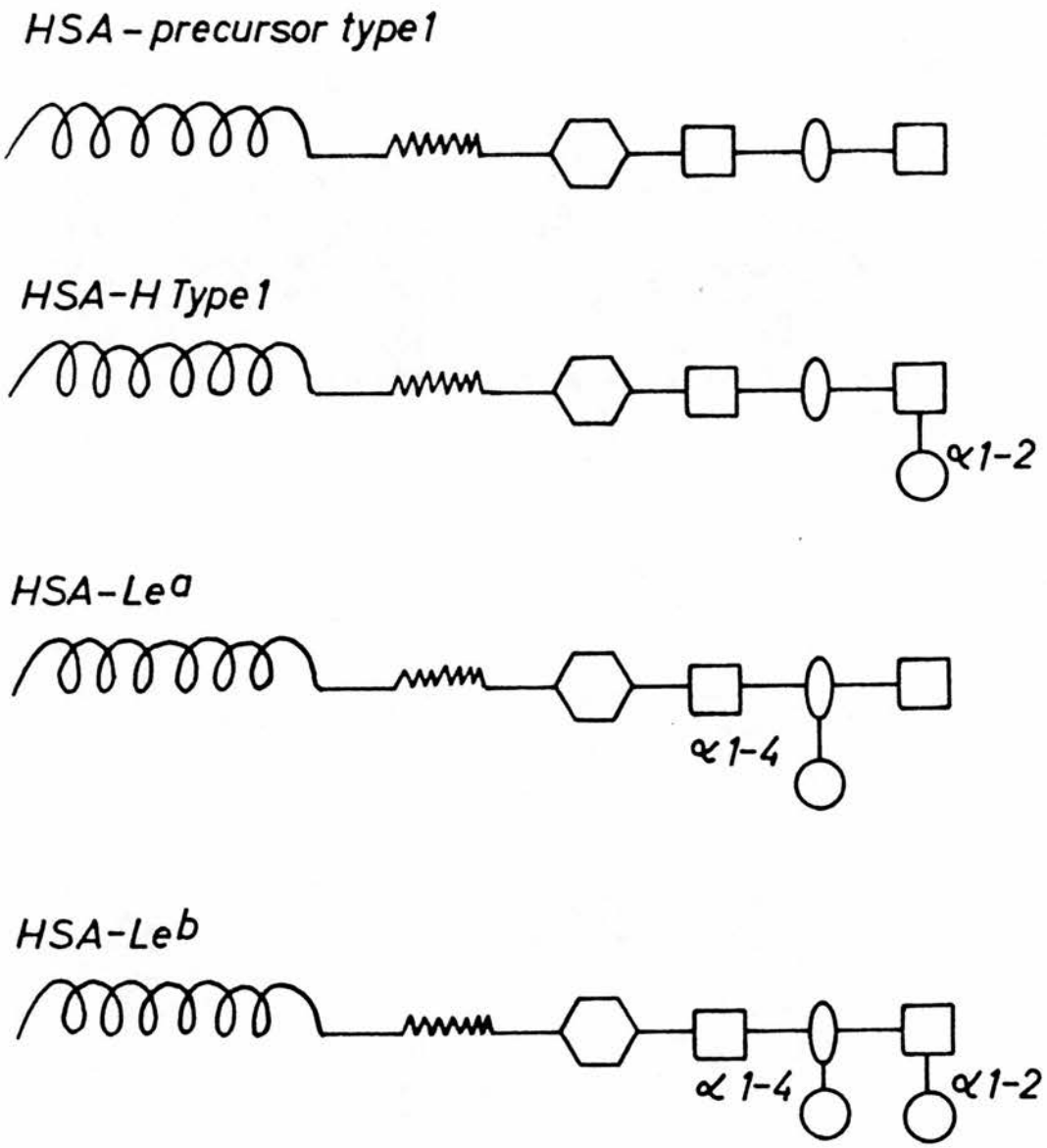


Figure 6.2: Purified blood group glycoconjugates used in the ELISA. Symbols as in Figure 1.1. The human serum albumin (HSA) backbone molecule is represented by. The acetylphenylenediamine molecule (APD) is a spacer used to conjugate the oligosaccharide chain to the HSA molecule. Each HSA molecule had an average of 10 to 20 oligosaccharide moieties.

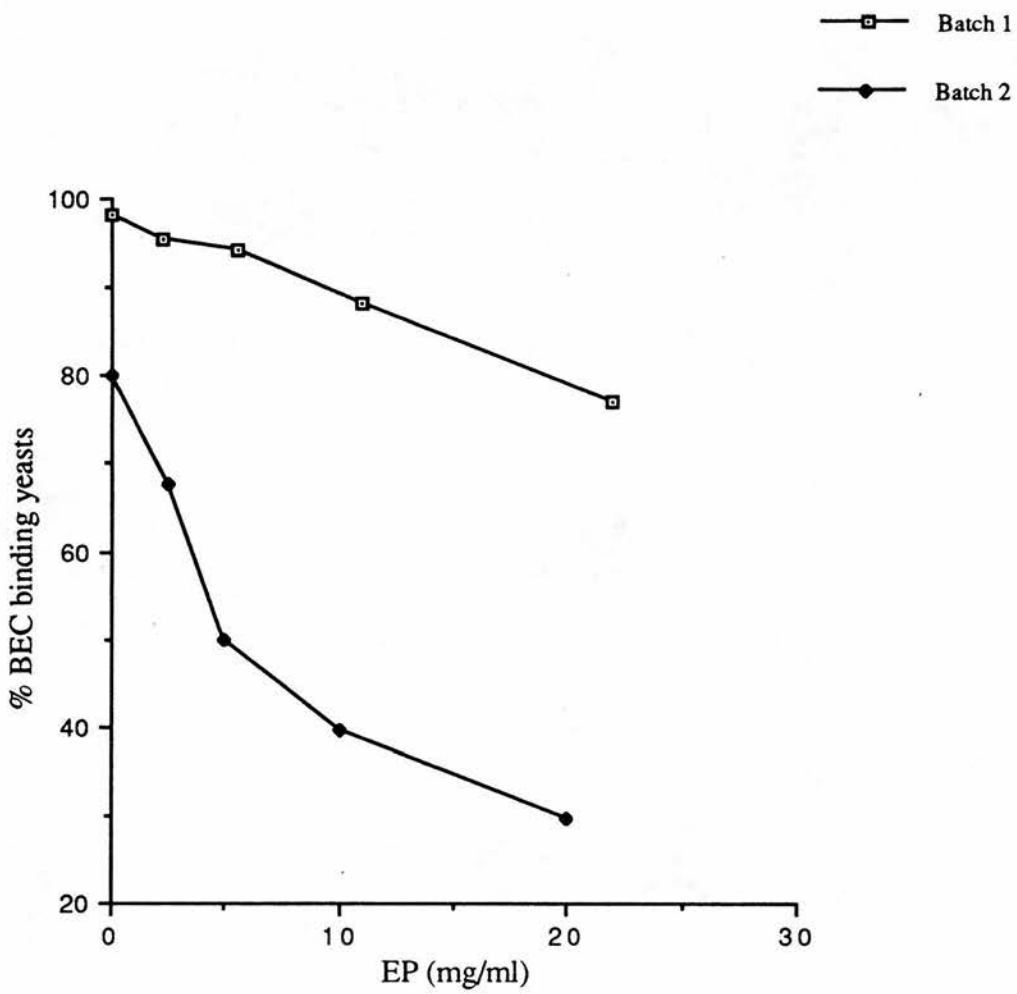


Figure 6.3: Inhibition of binding of *C. albicans* 2346 to 2.5 x 10^5/ml BEC pretreated with EP2346 expressed as % BEC binding yeasts

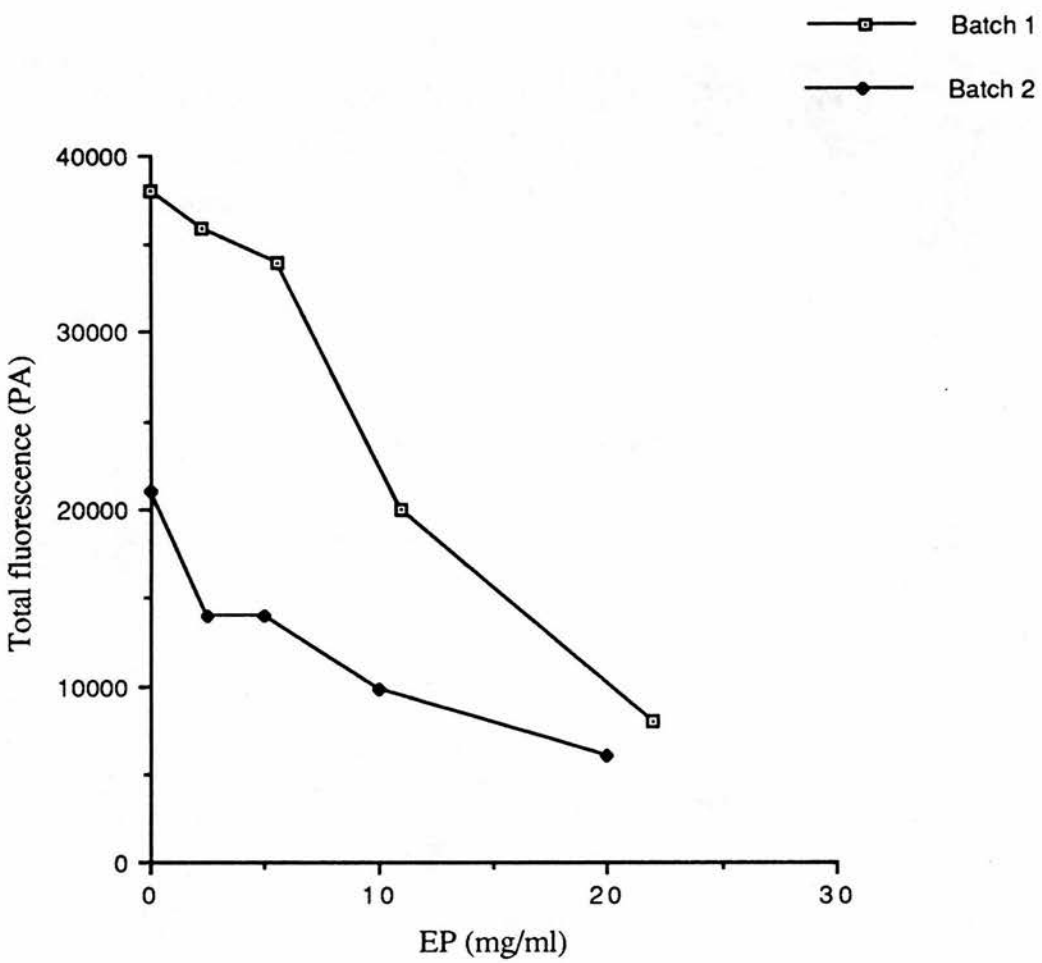


Figure 6.4: Inhibition of binding of *C. albicans* 2346 to 2.5 x 10^5/ml BEC pretreated with EP2346 expressed as total fluorescence (PA)

Table 6.1 Binding of glycoconjugates to EP 2346: ELISA reading

Batch	Protein (mg/ml)	Lewis[a]	Lewis[b]	H Type 1	Precursor
1	7.0	0.717			
2	6.8	0.403	0.317	0.321	0.364
3	6.1	0.228	0.154	0.167	0.204
4	6.5	0.325	0.291	0.276	0.301

*maximum ELISA reading was 2.00

Table 6.2 Inhibition of binding of *C.albicans* 2346 to BEC pretreated with EP 2346

Batch	EP 2346 (mg/ml)	% BEC	MFC	PA (10^3)	inhibition (%) PA	inhibition (%) BEC
1	11	88	228	2.0	47	10
2	10	40	246	9.8	53	50
3	10	36	221	7.9	50	53
4	10	46	160	7.3	57	57

Dynatech micro-plate reader MR 700 equipped with a tungsten halogen lamp (GE#787) at 490 nm. Control wells contained: 1) 40 ul of 1mg/ml of HSA (Sigma, Dorset, U.K) and 10 ul of blocking buffer; and, 2) 50 ul of PBS instead of the glycoconjugate.

6.3 Results

6.3.1 Protein content of EP_{2346}

Four separate 1 litre batches of 5 day cultures of yeasts were analysed for protein content. Table 6.1 shows that the protein content ranged from 6.1 to 7.0% comparable with the results of McCourtie and Douglas (1985) of 6.9 ± 0.2% protein determined by the Lowry method on one 1 litre batch of the growth culture.

6.3.2 The adherence inhibition activity of EP_{2346}

Each batch of EP was tested for the presence of inhibitory activity before use in the ELISA assay (Table 6.2). At a concentration of 10 mg/ml, all four batches of EP inhibited the binding of *C. albicans* 2346 to BEC by 47-59% as estimated by the PA values. When only the % of BEC binding *C. albicans* 2346 is considered, the inhibition at 10mg/ml of EP is more variable (10-57%). The differences are illustrated in Figures 6.3 and 6.4. There are obvious variations in the activity of the inhibitory components of different batches of EP; and, this difference was independent of the protein content of the EP.

6.3.3 Binding of glycoconjugates to EP

All batches of EP bound the HSA avidly and this reading was subtracted from the readings obtained from the other wells. All glycoconjugates carrying the blood group determinants bound to EP (Table 6.1). There was a consistent trend: the ELISA reading for the glycoconjugates with the Le^a determinant was highest followed by that with the precursor Type 1 determinant. There was no consistent trend for Le^b determinant compared with the H Type 1 determinant; the ELISA reading for both were lower compared with Le^a and precursor Type 1. As the protein content of the EP decreased, so did the readings obtained with the Le^a glycoconjugates; however,

this decrease was not proportional to the decrease in protein content.

6.4 Discussion

Binding of the yeasts to BEC pretreated with EP was carried out to determine if the inhibitory activity of the EP was intact. The EP batches were not identical in their inhibitory activities. This is illustrated in Figures 6.3 and 6.4 in which the slopes of the graphs for batches 1 and 2 are different. This might be due to an absolute difference in the inhibitory component and/or the variation in the BEC from the single donor on different days. The latter possibility could account for the greater part of the variation as is seen from the wide range of inhibition results (10-57%) when only percentage of BEC inhibited are examined. It can also be concluded that the PA scale provides a better and less variable (47-59%) representation of the results of the adhesion assay; the slopes in Figure 6.4 are almost parallel compared with those in Figure 6.3. Other investigators have suggested that measurement of mean number of bacteria attached to the entire host cell population provided a more accurate estimate of adhesiveness (Freter and Jones, 1983). The adhesion assay was not affected by subjective reading errors; therefore, this aspect was not responsible for the variation in the slopes of the graphs.

The PA figures compare well with the results of other studies in which the adhesion assay was carried out by light microscope to quantitate inhibition of binding of yeasts to BEC pretreated with EP; 50% inhibition was achieved with 10 mg/ml EP in two experiments and with 5mg/ml in another (McCourtie and Douglas, 1985; Critchley and Douglas, 1987a; Critchley and Douglas, 1987b).

In the ELISA assay, there was a clear trend for binding of blood group determinants to EP; ELISA readings were highest for Le^a followed by those for precursor Type 1 determinant. The readings for H Type 1 and Le^b determinants were consistently lower compared with Le^a, possibly because the fucose molecule was in a less favourable conformation. The precursor Type 1 determinant, which has no fucose molecules, bound to the EP. This might be a due to other adhesins present in the EP reacting with the precursor Type 1 structure and/or due to more effective binding of adhesin(s) in the EP to sites that are unmasked as a result of the absence of fucose molecules. Differences in the strength of bacterial binding to glycoconjugates with different blood group determinants on a Type 1 precursor chain have been reported (Rosenstein *et al.*, 1988). Modification of the precursor structure by addition

of monosaccharide (in H and Le^b determinants) interfered with binding of *E. coli*.

In general the results of the ELISA assay complement those of the adhesion assay (Chapter 5). Cells from non-secretors which should have more Le^a on their surfaces had the highest PA values than those from secretors; and, the two Le^{a-b-} individuals had the lowest PA values. *C. albicans* 2346 was also found to co-agglutinate Sepharose-Protein A beads to which anti-idiotype antibody to monoclonal anti-Le^a was conjugated. In contrast, *C. albicans* 2023 did not co-agglutinate the Sepharose beads (S. Essery, personal communication). This strongly suggests that *C. albicans* 2346 can bind to Le^a-like structure.

A recent study reported that the *C. albicans* fucose-containing lectin could be isolated by an affinity system containing the H determinant (Douglas, 1991). The material eluted from the column inhibited adherence of BEC 220 fold more efficiently than crude EP; experiments with Le^a were not done. This complements the results of the ELISA assay in which binding of EP to glycoconjugate containing the H determinant was demonstrated; however, it predicts that the Le^a determinant is probably a more efficient receptor than the H determinant.

Another recent publication reported that human milk oligosaccharide could inhibit the binding of *C. albicans* 17113 to BEC. The yeast has fucose-recognising adhesins and the minimal structure requirement for the inhibitory activity was found to be Fucα1 $\rightarrow$ 2Galβ (H determinant) which is present on both H Type 1 and H Type 2. In contrast, the fucosylated determinants of the Lewis blood group system were found to be totally inactive (Brassart *et al.*, 1991). Although the H determinant inhibited an adhesin on this particular strain, H Type 1 is not present on cells or in secretions of non-secretors; therefore this mechanism cannot explain the increased binding of *C. albicans* 2346 to non-secretor cells. There is an additional argument against H Type 1 playing a role *in vivo*. In most populations >95% of secretors have the *Le* gene; therefore, most of the H Type 1 will be fucosylated by the product of the *Le* gene to Le^b, ALe^b or BLe^b.

Chapter 7
General Discussion and Conclusions

This chapter summarizes the findings and conclusions presented in the previous chapters in the context of the objectives stated in Chapter 1.

7.1 Epidemiology of oral disease and carriage of yeasts

Diabetics are immunocompromised individuals prone to oral and vaginal candidiasis. In this study, denture stomatitis was used as a model of superficial candidiasis. The objective of the epidemiological study was to identify the factors predisposing to carriage of yeasts and to denture stomatitis.

Previous epidemiological studies of diabetic individuals did not use multivariate statistical analyses, some did not differentiate IDDM from NIDDM and some examined small numbers of patients. This meant that factors analysed by univariate analysis could be obscured by other dependent variables. In this study, eighteen variables were analysed for their contribution to carriage of yeasts and development of denture stomatitis: gender; age; type of diabetes; diabetic treatment (insulin, biguanides, sulphamideureas, dietary); duration of diabetes; HbA_1; random plasma glucose; diabetic complications (retinopathy, peripheral neuropathy, autonomic neuropathy, nephropathy); antibiotic treatment; corticosteroid usage (local and systemic); smoking; alcohol consumption; dentures (extension, occlusion, fit, hygiene, continuous wear); ABO blood group; secretor status; glycosuria; albuminurea and past history of candidiasis. The variables with p values < 0.01 isolated by the multivariate analysis were, in most cases, also those that were significant in the univariate analysis. The advantage of the multivariate analysis was that it detected marginally significant variables ($p < 0.05$) such as plasma glucose, retinopathy and non-secretion of blood group antigens not isolated by the univariate analysis.

The multivariate analysis also indicates the prognostic value of the isolated variable(s). Among patients with NIDDM a combination of continuous presence of a denture ($p < 0.01$), increased number of yeasts ($p < 0.01$) and non-secretion of blood group antigens ($p < 0.01$) were used to predict correctly 82% of subjects with denture stomatitis. Persistent glycosuria ($p < 0.05$) was not a good indicator of denture

stomatitis among subjects with IDDM (Table 4.6) because it could only predict 63% of the patients with denture stomatitis. This suggests that variable(s) other than those investigated contribute to development of denture stomatitis among individuals with IDDM.

Palatal carriage of yeasts was correctly predicted by glycosuria ($p < 0.01$) as the sole predicting variable in 78% of NIDDM carriers who did not wear dentures. It is tempting to assume that variables with lower p values would be better predictors; however, this is not the case. Among individuals with IDDM who wore dentures the analysis correctly predicted only 71% of palatal carriers from three factors: HbA_1 levels ($p < 0.01$), age ($p < 0.05$) and random plasma glucose levels ($p < 0.05$).

The epidemiological studies showed that risk factors influencing carriage of and disease due to yeasts were distinct among individuals with IDDM compared with those with NIDDM (Tables 2.9, 2.10 and 4.6). Future studies examining other factors such as the role of vitamins and immune system functions can be analysed in a similar manner to reveal their contribution towards colonization and disease.

7.2 Studies on adherence of *C. albicans* 2346 to BEC

The second aim of the study was to investigate the reasons underlying the increased susceptibility of non-secretors to oral disease due to yeasts. The adherence of *C. albicans* 2346 to BEC from secretor and non-secretor individuals was compared. Epithelial cells from secretors bound fewer *C. albicans* 2346 than BEC from non-secretors. Cells of blood group O individuals were used to eliminate interference by other blood groups such as blood group A which is known to be a receptor for some strains of *Candida* (Tosh and Douglas, 1991). The number of individuals sampled was limited by the availability of non-secretors who make up approximately 25% of the population. Despite examination of over 2000 BEC per individual assay, the significance value for the difference between secretors and non-secretors did not achieve $p < 0.05$. The results agree with those of Douglas and Tosh (1991) obtained in studies in which light microscopy was used to determine binding of *C. albicans* 2346. BEC from blood group O and A non-secretors bound more yeasts than those from secretors. In their study, these differences were significant probably due to their small sample size (number of donors not given); secretor donors with very low levels of Le^a might have been included in their experiments.

Two important points arise from these observations. First, binding assays with epithelial cells will need to take into consideration the amount of the putative receptor on the cells of secretors and non-secretors. Second, the higher binding of *C. albicans* 2346 to non-secretor cells cannot be due to differences in H antigen on secretor and non-secretor cells. Non-secretor and secretor cells express similar quantities of H Type 2 antigen (Rahat *et al.*, 1990). The cells of secretors bind significantly greater amounts of *Ulex europaeus* lectin; this is due to H Type 1 which non-secretors are incapable of producing. Additionally, H Type 1 is a substrate for the product of the *Le* gene that converts it to Le^b *in vivo*.

In this context, the conclusions of a recent study in which human milk oligosaccharide probes were used to inhibit the binding of *C. albicans* 17113 to BEC need to be reassessed. The yeast has fucose-recognizing adhesins and the minimal structure requirement for the inhibitory activity was found to be Fucα1 $\rightarrow$ 2Galβ (H determinant) which is present on both H Type 1 and H Type 2 chain structures. The determinants of the Lewis blood group system which are fucosylated at the subterminal position of the Type 1 precursor chains were found to be totally inactive (Brassart *et al.*, 1991). While the *in vitro* studies appear to be valid, the authors have not considered the conditions that prevail *in vivo*.

7.2.1 Le^a as a putative BEC receptor for *C. albicans* 2346

Since BEC from non-secretors bound more yeasts compared with cells from secretors, the role of Le^a antigen as a receptor for the fucose-recognising adhesins of *C. albicans* 2346 was explored. Experiments were designed to examine the ability of yeasts to adsorb $Lewis^a$ from saliva. Serial dilutions of partially purified Le^a antigen adsorbed with the yeasts were analysed for the presence of Le^a by an ELISA method (Raza *et al.*, 1991). Results of this experiment were inconclusive because compared with the unadsorbed control, a higher reading of Le^a was obtained from solutions adsorbed with the yeasts. It was suggested that there might be cross-reaction between *C. albicans* 2346 antigens and the Le^a antigen detected by the monoclonal anti-$Lewis^a$ antibody; and, *C. albicans* was secreting or sloughing the cross-reactive antigens into the solution.

A different method was, therefore, developed to test whether the fucose-recognising adhesin on *C. albicans* 2346 bound Le^a. Binding of purified glycoconjugates to EP from *C. albicans* 2346 showed that the highest readings in the

ELISA were obtained with the Le^a glycoconjugate. The other glycoconjugates also bound to the EP, but the ELISA readings were lower. These results are compatible with other published accounts indicating that blood group antigens, and especially Le^a might be receptors for yeasts *in vivo* (Thom *et al.*, 1989; May *et al.*, 1989).

The Le^a determinant on host cells is considered to be the most likely receptor for the fucose-recognising adhesin of *C. albicans* 2346. The higher binding of Le^a glycoconjugate to EP 2346 compared with those containing Le^b or H Type 1 determinants help to explain the increased colonization of non-secretors. The amount of anti-Lewis^a antibody bound to BEC has been shown to vary among individuals. In flow cytometry assays, consistently high levels of anti-Lewis^a antibodies were bound to cells of non-secretors; and, consistently low levels of the antibody were bound to cells from $Le^{a-,b-}$ individuals. Cells from secretors, however, bound variable amounts of anti-Lewis^a antibodies. Cells from some secretor donors bound as much anti-Le^a antibody as cells from non-secretors; for other secretor donors, the levels were as low as those for $Le^{a-,b-}$ cells (Blackwell *et al.*, 1992, in press). The presence of variable amounts of Le^a on cells of individuals who are secretors might account for the high standard error found in the adhesion assay and the inability to achieve a p value of ≤ 0.05 in the present study in which secretor/non-secretor pairs were selected only by age and gender but not the amount of Le^a on their cells.

7.3 Pathogenesis of denture stomatitis among individuals with diabetes mellitus

The mechanisms underlying the development of superficial candidiasis remain obscure. In non-diabetic individuals, carriage of yeasts is a stable characteristic over a period of time (Burford-Mason *et al.*, 1988). A scheme of carriage leading to denture stomatitis was proposed by Samaranayake and MacFarlane (1985). The presence of a denture created an environment in which yeast virulence factors such as proteinases act. Sucrose from dietary sources helps in the establishment and proliferation of the yeasts. Production of acidic metabolic waste products would allow yeast proteinases to act as low grade aggressins. The host will react to the insult by producing an inflammatory response. This sequence of events does not contradict the histological appearance of the disease process.

Except for the increased levels of glucose, conditions leading to the development of denture stomatitis in individuals with diabetes mellitus have not been considered

before in any detail. In this section, two models for colonization by yeasts and development of denture stomatitis are proposed based on the different risk factors identified in these studies for patients with IDDM compared with those with NIDDM.

Factors influencing palatal colonization of individuals with NIDDM who did not wear dentures were persistent glycosuria, and, for denture wearers, the continuous presence of the denture in the mouth. Development of denture stomatitis was influenced by a combination of continuous wear of denture, increase in the density of colonization and non-secretion of blood group antigens.

High levels of glucose reflected by persistent glycosuria result in an increase in nutrients and adhesiveness of yeasts. Introduction of a denture and its continuous wear provides a microenvironment whereby proliferation of yeasts might occur. In the protected microenvironment under the denture, there is no cleansing action of saliva and yeasts might accumulate. The anaerobic growth conditions will result in the production of acidic metabolites. Yeast proteinases might be activated in this acid environment and act as low grade aggressins to induce inflammation. For those strains such as *C. albicans* 2346 which might use Le^a as a receptor, the epithelial cells might bind more yeasts initially and would be more densely colonized.

Other indirect mechanisms by which secretor status might influence disease include differences in the secretory immune responses of secretors and non-secretors. Non-secretors have lower levels of total salivary IgM and salivary IgM specific for *Neisseria* species compared with secretors (Zorgani *et al.*, 1992).

Factors influencing palatal colonization of individuals with IDDM who did not wear dentures were age; and, for denture wearers HbA_1 levels, age and random plasma glucose levels. Development of denture stomatitis was influenced by persistent glycosuria.

Colonization is increased among the relatively younger individuals. The increased colonization with decreasing age has not been reported before. This finding might be a unique feature of this population; but, an alternative hypothesis is suggested. Secretory IgA levels against *Candida* might increase with increasing age and increased frequency of exposure to *Candida*. Studies of specific secretory antibodies in this population will provide evidence to refute or support this hypothesis.

Introduction of a denture alters the colonization risk factors. Glycaemic control

(HbA_1) is most important followed by age and plasma glucose levels. As with patients with NIDDM, poor glycaemic control probably predisposes to colonization by increased availability of glucose as a nutrient. Development of denture stomatitis is precipitated by very poor control where persistent glycosuria is evident.

This is the first study to provide definite links between glycaemic control and development of denture stomatitis. This was found only for individuals with IDDM. In addition to effects on growth and acid production, there are other mechanisms by which increase in glucose levels might influence development of disease among patients with IDDM. They have lower levels of C3 (Charlesworth *et al.*, 1987) and binding of glucose to the biochemically active site of the C3 inhibits the attachment of this protein to the microbial surface and thereby impairs opsonisation (Davidson *et al.*, 1984). Yeasts can also bind complement reaction products by Complement Receptor Proteins (CRP) which are analogous to the human CRP. The yeast CRP has been shown to be induced by increased glucose level. Phagocytic (Davidson *et al.*, 1984) and killing ability (Wilson and Reeves, 1986) of granulocytes from diabetic individuals is reduced with increases in glucose levels. Yeast secretory proteinase is also induced by glucose levels; and, can act as a low grade aggressin (Ruchel, 1991). These factors might act synergystically to promote development of disease.

7.4 Future Aims and Investigations

Adhesion assays are limited because of variability of epithelial cells. Since recent studies have found a significant correlation between binding of anti-Lewis^a and binding of *Staphylococcus aureus* to BEC from secretors and non-secretors (Saadi *et al.*, submitted for publication), future binding studies with *C. albicans* 2346 need to control for this variable. Binding of yeasts to H Type 1 and Lewis antigens coupled to inert particles such as latex might provide clearer results. Other experiments which might elucidate the role of Le^a are immunoblotting techniques whereby yeast cell wall antigens separated by gel electrophoresis and blotted onto nitrocellulose could be tested for binding to purified blood group antigens. Experiments similar to that by Douglas (1991) with synthetic H determinant (Synsorb) might be undertaken to assess the binding affinity of the adhesin for different blood group determinants.

The obvious question at the end of this section is that of the practical applications of the study. The study has shown the advantages of using multivariate analysis in epidemiological studies. With reference to management of patients with denture

stomatitis, among individuals with IDDM improvement of glycaemic control should be strongly recommended; and, among those with NIDDM the denture should not be continuously worn. Correct identification of yeast species is also important from the therapeutic aspect of treatment. *C. albicans* accounts for just over half of the yeast isolates from denture stomatitis lesions; other species of *Candida* are important pathogens in this population. The API kit should not be relied on to identify *C. albicans*; the germ tube test should be used to confirm the presence of this isolate. Other *Candida* species are best identified by conventional methods described by Milne (1989).

The studies on identification of the fucose-containing receptor on host cells have long-term potential towards development of vaccines against *Candida*. Researchers involved in the development of vaccines for meningococcal meningitis have accepted that a cocktail vaccine is needed since different strains of the bacteria have different immunogenic determinants. The identification of the fucose-containing receptor(s) for the EP of *C. albicans* 2346 would be an initial stage leading to isolation of the adhesins and assessment of their immunogenicity. If the adhesin is non-immunogenic, this would be excluded from further studies to develop a vaccine. If there is evidence that antibodies to the adhesin are associated with decreased density of colonization or protection from denture stomatitis, it might be included as part of a vaccine for individuals at risk of candidiasis such as non-secretors who are diabetics and individuals in the early stages of AIDS.

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Appendix

1 Buffers

All chemicals were analytical grade obtained from BDH Chemicals Ltd. (Poole, Dorset, UK) or Sigma (Poole,Dorset, UK).

1.1 1M Carbonate-bicarbonate buffer (pH 8.9)

Stock solution of 1M sodium carbonate and 1M sodium bicarbonate were prepared. The buffers were mixed in the proportion 1:9 (v/v) and adjusted to pH 8.9 with one of the stock solutions (carbonate raises pH, bicarbonate lowers pH). The bicarbonate solution was prepared fresh for each experiment.

1.2 Dulbecco's phosphate-buffered saline, solution A (DPBS)

This buffer consisted of the following: 136 mM sodium chloride, 2.6 mM potassium chloride, 8.1 mM sodium hydrogen phosphate, 1.47 mM potassium dihydrogen phosphate, 0.2% aqueous phenol red (pH 7.3).

1.3 Dulbecco's phosphate-buffered saline with supplement B

This was prepared from DPBS by the addition of 0.9 mM Ca^{++} and 0.4 mM Mg^{++} ions.

1.4 Phosphate-buffered saline (PBS)

This was prepared by the addition of 0.01 M phosphate buffer pH 7.2 to 0.15 M NaCl.

1.5 Phosphate citrate buffer (PCB)

Sodium hydrogen phosphate at 0.1 M and 0.1 M citric acid solutions were mixed and the pH adjusted to pH 5.

1.6 Coating buffer

Coating buffer was composed of 15 mM sodium carbonate, 35 mM sodium bicarbonate and 3 mM sodium azide (pH 11).

1.7 Washing buffer

Washing buffer was prepared by adding gelatin (0.03% w/v) and Tween-20 (0.05% v/v) to 0.01 M PBS (pH 7.2).

1.8 Blocking buffer

This was composed of 0.01 M PBS containing 0.3% (w/v) gelatin (pH 7.2).

1.9 Substrate solution

The substrate solution used in the colour development reaction with horseradish peroxidase contained 40 mg O-phenylenediamine in 100 mM phosphate citrate buffer activated immediately before use by the addition of 40 ul H_2O_2 (30% v/v).

2 Culture Materials

2.1 Malt broth

Made up from 20 g malt extract (desiccated) in 1 litre of water. Steam was used to aid dissolution and the solution adjusted to pH 5.4. Sterility was obtained by autoclaving at 120 °C for 20 minutes. Aliquots of 5 ml were dispensed into sterile bijou bottles.

2.2 Cornmeal agar

This medium was prepared from commercially available dehydrated ingredients (Oxoid) according to the manufacturer's instructions. The ingredients present in the dehydrated medium were: 2g K_2HPO_4, 1g glucose, 1g peptone and 5g NaCl. This

was dissolved in 1 litre of distilled water by steaming. The pH was adjusted to 6.8-6.9 before the addition of 6 ml of 0.2% phenol red solution. Agar (20 g) was added and the mixture autoclaved at 115 °C for 15 minutes. After sterilization, 40 mls of 50% urea was added and allowed to cool to 50 °C before pouring into Petri dishes. The pH of the medium was 6.0 ± 0.2.

2.3 Christensen's urea agar containing glucose

This contained 5g of glucose and 5g of sodium chloride.

2.4 Malt agar plates

Contained 30g desiccated malt extract (Oxoid) , 5g mycological peptone and 15g of agar dissolved in 1 litre of distilled water. The pH was adjusted to pH 5.4 by addition of lactic acid and autoclaved at 115 °C for 10 min before being dispensed into Petri dishes.

2.5 Yeast nitrogen extract (Difco) with 500 mM galactose

A ten times strength solution was prepared by dissolving 5 M galactose in 100 ml distilled water and addition of 6.7 g of Bacto yeast nitrogen base. The solution was filter sterilized and diluted with distilled water before use (pH 5.4 ± 0.1).

2.6 Buffered paraformaldehyde

Sodium calcodylate (10 g) was added to 1 litre of distilled water and adjusted to pH 7.2. Paraformaldehyde (10 g) was added and dissolved by boiling the solution. Finally, 7.5 g sodium chloride was added and the solution stored at 4 °C.

2.7 *Candida albicans* strains

C. albicans 2346 and 2023 were obtained from Dr. J. Douglas, Department of Microbiology, University of Glasgow. These strains were isolates from patients with denture stomatitis. *C. albicans* 2346 had a fucose-recognising adhesin and was used as a test strain in the adhesion assays. *C. albicans* 2023 had an

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Chronic atrophic oral candidiasis among patients with diabetes mellitus – role of secretor status

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SUMMARY

Non-diabetic individuals who are non-secretors of blood group antigens are prone to superficial infections by *Candida albicans*. In this study, 216 patients with diabetes mellitus who were denture wearers were examined for the presence or absence of denture stomatitis. There was an overall trend for non-secretors to be prone to denture stomatitis compared with secretors. Stepwise linear discriminant analysis was used to dissect the contribution of secretor status and other variables to the development of the disease. Secretor status was found to be a contributory factor among patients with non-insulin dependent diabetes but not among those with insulin-dependent diabetes. The possible reasons for this are discussed.

INTRODUCTION

Chronic atrophic oral candidiasis (denture stomatitis) is solely associated with the wearing of dentures. The studies of Butdz-Jörgensen [1] and Cawson [2] established the link between *Candida* sp. and denture stomatitis. Yeasts were recovered from over 90% of the lesions and antifungal treatment usually led to regression of the lesion. Subsequently, numerous studies have examined the prevalence of and predisposing factors to denture stomatitis [3]. Presence and continuous wearing of dentures at night, availability of sucrose in the oral environment through dietary intake, traumatic effects of the denture, antibiotics and corticosteroid treatment, diabetes mellitus and smoking have all been implicated as potential factors influencing the development of denture stomatitis.

The genetic inability to secrete ABO blood group antigens in body fluids has been associated with a variety of infectious diseases [4]. Non-diabetic individuals and pregnant women who are non-secretors are prone to superficial candida infections [5]. Non-secretors are also over-represented among carriers of *Candida albicans* in normal subjects and patients with non-insulin dependent diabetes mellitus (NIDDM) [6, 7].

The aims of the present study were:

(1) To assess the influence of secretor status in the development of denture stomatitis (DS) among patients with insulin-dependent diabetes (IDDM) and those with non-insulin dependent diabetes (NIDDM).

(2) To assess the contributions of the following variables to the development of DS: age; sex; duration of diabetic state; type of diabetes; control of diabetes measured by glycosylated haemoglobin (HBA_1); diabetic complications – retinopathy, neuropathy and nephropathy; antibiotics; corticosteroid treatment; smoking; alcohol; presence of dentures at night; type of denture; denture fit, extension, occlusion, age and hygiene; presence of persistent glycosuria and albuminuria; and history of superficial candida infections.

MATERIALS AND METHODS

Subjects

A total of 439 subjects attending for routine follow up examination at the Diabetic Outpatient Department (DOPD), Royal Infirmary Edinburgh were sampled. An initial pilot study examined 80 individuals and was followed by a study that sampled 359 individuals between September 1988 and March 1989. The method of selection was stratified random selection according to sex and type of diabetes.

Clinical history

Each subject was classified as insulin dependent (IDDM) or non-insulin dependent (NIDDM) according to the clinical history of onset, requirement for insulin and progression of the disease. Of the 439 subjects sampled, three could not be classified.

A full medical history including the presence of diabetic complications (retinopathy, neuropathy and nephropathy) was obtained during interview and from the patients' records. A history of medications, with particular reference to antibiotics or corticosteroid-containing preparations, within the past 6 months was noted. A social history of alcohol consumption and smoking was recorded. Subjects were questioned about history of superficial infections due to candida. Glycosuria and albuminuria were recorded as persistent if subjects had positive urine samples on more than two consecutive appointments at the DOPD. None of the subjects used any oral preparations containing antiseptics within the previous 6 months.

Clinical examination

A thorough oral examination of both soft and hard tissues was carried out by the same examiner (FZA). Any of the following abnormalities were noted: gingivitis, periodontitis, angular cheilitis, leukoplakia, median rhomboid glossitis; fissured, geographic or hairy tongue; and denture stomatitis. Denture stomatis has the characteristic appearance of chronic erythema of the portion of the palate underlying the denture. The inflammation is generally diffuse, but may be associated with fibrous hyperplasia of the palate giving it a granular appearance. The occlusion, fit, extension and hygiene of a denture where present was recorded

as 'good' or 'poor'. The age of the denture was recorded as well as whether it was left out of the mouth at night.

Samples

(1) Venous blood was obtained for ABO blood grouping and Lewis antigen determination. Analysis for glycosylated haemoglobin (HBA_1) and random plasma glucose carried out routinely were recorded.

(2) Swabs were obtained from five sites of the mouth including the palate and inoculated immediately into malt broth.

(3) Subjects were requested to rinse with 10 ml of sterile phosphate-buffered saline (PBS) for 1 min and to return the contents to a sterile container.

Laboratory analysis

All samples were collected between 09.00 and 12.00 h and processed within 1–2 h. ABO blood group was determined by slide agglutination with monoclonal anti-A and anti-B antibodies (Scottish National Blood Transfusion Service). Secretor status was determined by the presence of Lewis antigen on red blood cells by tube agglutination with monoclonal anti-Lewis^a and anti-Lewis^b antibodies (Scottish National Blood Transfusion Service). The haemagglutination inhibition method with boiled saliva [8] was used to confirm the Lewis antigen results for 159 individuals.

The swabs were incubated at 37 °C for 36–48 h, plated onto malt agar and incubated for a further 36–48 h.

The mouth rinse was concentrated by centrifugation at 2000 ***g*** and resuspended in 1 ml of PBS; 20 μl of the suspension were inoculated onto malt agar plates and incubated at 37 °C for 36–48 h. The number of colonies per sample were recorded.

Pure colonies were subcultured and identified with the API 20C AUX. Additionally all were identified by the conventional methods [9] of germ-tube production in horse serum, urease test and hyphae production on corn-meal agar following incubation at 28 °C for 48 h.

Statistical methods

All results were coded and a computerized database was set up to facilitate analysis using SPSSX. The relationship between prevalence of DS and other factors was tested by χ^2 (with Yates' correction) or Wilcoxon rank sum tests. Stepwise linear discriminant analysis was used to identify which combinations of factors best predicted the presence or absence of DS.

RESULTS

Oral conditions among the patients examined are summarized in Table 1. Of the 216 denture wearers, 76 (35%) had DS (Table 2). In 18% (14/76) the rinsing technique failed to isolate any yeasts. Similar results were obtained with the swab taken from the palate. The species most frequently isolated from the palate of patients with DS were *C. albicans* (33/76, 43%), followed by *Torulopsis glabrata* (6/76, 8%) and *C. tropicalis* (3/76, 4%). In 9%, the isolate could not be identified to a species level. Other species were isolated in 15% (11/76) of DS cases (Table 3).

Table 1. *Prevalence of oral conditions among diabetic individuals*

	Prevalence in total population screened	Prevalence among denture wearers
Gingivitis	114 (28%)	39 (42%)
Angular cheilitis	42 (10%)	25 (12%)
Denture stomatitis	76 (35%)	76 (35%)
Fissured tongue	25 (6%)	15 (7%)
Dry mouth	15 (3%)	13 (6%)
Geographic tongue	11 (3%)	7 (3%)
Median rhomboid glossitis	10 (2%)	7 (3%)
Hairy tongue	8 (2%)	5 (2%)
Glossitis	7 (2%)	5 (2·3%)
Leukoplakia	2 (0·5%)	2 (1%)

Table 2. *Prevalence of DS among patients with IDDM or NIDDM*

	+DS	−DS	Total
IDDM	32 (41%)	47 (59%)	79 (100%)
NIDDM	43 (32%)	91 (68%)	134 (100%)
	75	138	213

Missing, 3; $\chi^2 = 1{\cdot}20$; $P = 0{\cdot}27$.

Table 3. *Mycological profile of diabetic individuals* (*Palatal swab*)

Isolate	Denture wearers no. (%)	DS cases no. (%)
C. albicans	67 (31)	33 (43)
T. glabrata	13 (6)	6 (8)
T. beigelli	5 (2)	3 (4)
C. tropicalis	4 (2)	3 (4)
C. paratropicalis	3 (1)	2 (3)
S. cereviciae	2 (1)	0 (0)
C. stellatoidea	1 (0·5)	1 (1)
T. inconspicua	2 (1)	1 (1)
C. luscitaniae	1 (0·5)	0 (0)
C. humicola	1 (0·5)	0 (0·5)
C. pseudotropicalis	1 (0·5)	1 (1)
Unidentified	24 (11)	7 (9)
Missing	5 (2)	2 (3)
Other	4 (2)	3 (4)
No isolate	83 (38)	14 (18)
Total	216	76

Secretor status

There was a trend for non-secretors to be prone to DS (Table 4). Among individuals of blood group O with DS, non-secretors (13/27, 48%) appeared to be more prone to DS than secretors (19/68, 28%); $\chi^2 = 2{\cdot}69$, D.F. = 1, $P = 0{\cdot}1$. This was not seen among the 73 blood group A individuals. There were 10 (45%) A non-secretors compared with 15 (29%) A secretors with DS ($\chi^2 = 1{\cdot}1$, D.F. = 1, $P = 0{\cdot}3$).

Table 4. *Secretor status and prevalence of DS among patients with diabetes*

	Denture stomatitis			
	Present no. (%)	Absent no. (%)	χ^2	P
Patient category				
Total ($n = 199$)				
Secretor	45 (31)	100 (69)	2·56	0·11
Non-secretor	24 (44)	30 (56)		
IDDM				
Secretor	18 (40)	27 (60)	0·03	0·87
Non-secretor	13 (45)	16 (55)		
NIDDM				
Secretor	27 (27)	72 (73)	1·90	0·17
Non-secretor	11 (44)	14 (56)		

Table 5. *Relationship between DS and presence of denture in the mouth at night*

		Denture stomatitis			
Patient category	Presence of denture	Present No. (%)	Absent No. (%)	χ^2	P
Total*	Yes	45 (52)	42 (48)	23·2	< 0·000 01
	No	7 (12)	53 (88)		
IDDM	Yes	18 (49)	19 (51)	3·50	0·062
	No	3 (18)	14 (82)		
NIDDM	Yes	27 (54)	23 (46)	18·82	< 0·000 01
	No	4 (9)	39 (91)		

* Of a total of 216 denture wearers, 43 were recruited during the pilot study and were not questioned about their denture wearing habits. For 26 individuals this information was not recorded during the main study period.

Significant associations were found between DS and the following:

Presence of dentures in the mouth at night. Significantly more individuals who did not remove their dentures at night (45) had DS compared with those who removed their dentures at night (7) ($P < 0·000 01$). This was found particularly among NIDDM individuals (Table 5).

Number of colonies isolated by the mouth rinse technique. Subjects who did not have DS had significantly fewer colony forming units (median = 10 c.f.u./ml) than subjects with DS (median = 1850 c.f.u./ml, $P < 0·0001$). This was seen particularly for patients with NIDDM (median = 1500 c.f.u./ml with DS and 25 c.f.u./ml without DS, $P = 0·0003$). Those with IDDM did not show this relationship (median = 2400 c.f.u./ml with DS, 502·5 c.f.u./ml without DS, $P = 0·07$).

Random plasma glucose. Lower values for random plasma glucose were observed among the 132 patients without DS (median = 9·55 mmol/l) than those observed for the 72 patients with DS (median = 10·95 mmol/l, Mann–Whitney $P = 0·02$). This was seen particularly among individuals with NIDDM (median = 10·75 mmol/l with DS and 9·5 mmol/l without DS, $P = 0·04$). Individuals with IDDM

Table 6. *Stepwise discriminant analysis (Wilks)*

Sample	Cases predicted (%)	Variables isolated
IDDM ($n = 52$)	62·90 %	Glycosuria ($P < 0·05$)
NIDDM ($n = 88$)	81·82 %	Denture in at night ($P < 0·01$) No. of yeast colonies ($P < 0·01$) Non-secretion ($P < 0·01$)

did not show this relationship (median 11·250 mmol/l with DS and 10·15 mmol/l without DS, $P = 0·24$).

History of candidiasis. Among individuals with a history of superficial candidiasis 18/33 (55 %) had DS compared with 58/182 (32 %) with a negative history of candidiasis ($\chi^2 = 5·33$, D.F. = 1, $P = 0·02$). Separate analysis with respect to insulin dependency revealed a trend for individuals with a history of candidiasis to be more prone to DS: for IDDM individuals $\chi^2 = 1·2$, D.F. = 1, $P = 0·3$; for individuals with NIDDM, $\chi^2 = 3·26$, D.F. = 1, $P = 0·07$.

No significant association was found between DS and the following variables: sex; age; type of diabetes; control of diabetes (HBA_1); duration of diabetic state; ABO blood group; smoking; alcohol consumption; persistent glycosuria or albuminuria; denture fit, hygiene, occlusion, age or type (partial or full); presence of diabetic complications (neuropathy, nephropathy, retinopathy); contraceptive pill; antibiotics; and corticosteroids (systemic or topical).

In order to determine the relative contributions of variables in the development of DS, a stepwise discriminant analysis (SPSSX) was used. Initially, all variables were screened at an F value of 4 ($P < 0·05$). Analysis was then confined to the variables isolated as significant to increase the number of cases examined. Table 6 summarizes the contributory variables and the significance of their contribution in predicting infection. For patients with IDDM, the only variable identified by the analysis was persistent glycosuria ($P < 0·05$). For patients with NIDDM three factors were identified: denture present at night ($P < 0·01$), number of yeasts isolated ($P < 0·01$) and non-secretion ($P < 0·01$).

DISCUSSION

All forms of the oral conditions listed in Table 1 are more prevalent among the patients with dentures than those without dentures. Fissured tongue was encountered in 6 % of the diabetic individuals. This figure is similar to that reported for 2478 dental patients [10]. The prevalence of geographic tongue and median rhomboid glossitis was slightly higher among diabetics than among dental and dermatology patients [11]. All the above conditions were much lower in prevalence than those reported for diabetic individuals by Farman who examined coloured South Africans [12]. These differences might be due to genetic and/or environmental factors between the predominantly Northern European population sampled here and that sampled by Farman. Dry mouth was a spontaneous complaint in only 3 % of the samples compared to 34 % reported by Sharon and colleagues [13].

The univariate analysis showed that prevalence of DS was similar among patients with IDDM or NIDDM. The prevalence of DS reported (35·2 %) is within the range (24–60 %) reported for non-diabetic individuals [3].

C. albicans was isolated from only 43 % of the patients with DS. Isolation of *C. glabrata* (8 %), and other yeasts (28 %) from patients with DS indicate that, among diabetic individuals, species other than *C. albicans* are an important cause of disease.

Sex, denture trauma and hygiene [14–19], treatment with corticosteroids [21] and antibiotics [3], have variably been reported to be associated with DS among non-diabetic individuals. In this study, both the discriminant and univariate analyses did not reveal any association between these factors and DS.

Among patients with candida leukoplakia (CL), smoking is a significant factor in the pathogenesis of the lesion [20] which develops in sites away from the denture bearing area. In DS, smoking was not a factor in patients with either IDDM or NIDDM. This is consistent with the fact that the palate is protected from the effect of the smoke by the denture.

Control of diabetes as measured by glycosylated haemoglobin (HBA_1) was not associated with DS. Although this was unexpected at first sight, it is well established that blood glucose levels and salivary glucose levels among diabetic individuals do not correlate [13].

Discriminant analysis indicates that factors contributing to development of DS among patients with NIDDM are clearly different from those with IDDM. Patients with NIDDM who do not remove their denture at night, harbour a large number of yeasts and non-secretors of blood group antigens are particularly at risk of developing DS. In contrast, none of these factors influence the development of DS among individuals with IDDM. This is further supported by the results of the univariate analysis in which the P value for these factors among IDDM individuals are consistently higher than the P value among NIDDM individuals.

Although secretor status did not show a significant univariate association with DS among individuals with NIDDM, it was found to be significant in the multivariate analysis when adjusted for denture wearing habits and density of yeast colonization. This suggests that the univariate relationship might have been obscured by the other two contributory factors.

Persistent glycosuria was the only predictor of DS development among IDDM individuals. The presence of 11·1 mmol/l or more of glucose in the arterial blood results in the appearance of glucose in the urine. It was unexpected that neither HBA_1 nor random plasma glucose were implicated as both are more precise indicators of glucose availability than glycosuria. The possibility than glycosuria was a chance isolation cannot be dismissed especially since the significance of this variable was marginal. It appears that the discriminant analysis was not effective in identifying variables important to the development of DS among IDDM individuals.

Non-secretion of blood group antigens was a significant contributory factor among NIDDM but not among IDDM individuals. Non-secretion has been associated with carriage of yeasts among non-diabetic individuals [6] and patients with NIDDM [7]. This complements the results relating to the development of DS in these patients. It has been suggested that patients with IDDM are more

immunocompromized than NIDDM so that any protective effect of secretion of blood group antigens does not make a significant contribution to prevention of colonization or disease. A previous study [22] did not find an association between secretor status and DS among diabetics; however, the number of patients examined was much smaller, there was no differentiation between IDDM and NIDDM individuals and multivariate analysis was not applied to the data.

Identification of factors contributing to the development of DS among individuals with IDDM and NIDDM might have implications for treatment of this condition. Treatment of DS among patients with NIDDM might be similar to that in non-diabetic individuals [23], i.e. removal of denture, especially at night, which also reduces the density of colonization by yeasts. Among patients with IDDM, treatment of DS seems likely to depend on improvement of poor diabetic control reflected in persistent glycosuria.

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FACTORS INFLUENCING ORAL CARRIAGE OF YEASTS AMONG INDIVIDUALS WITH DIABETES MELLITUS

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Running head : Oral yeast carriage among diabetics

Key words : Candida albicans, diabetes, secretor status

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SUMMARY

A total of 439 individuals with diabetes mellitus were examined for carriage of yeasts by the oral rinse and palatal swab technique. The differences between the two techniques are discussed. Factors contributing to carriage of yeasts are distinct for individuals with insulin-dependent diabetes mellitus (IDDM) compared with non-insulin dependent diabetes (NIDDM). Introduction of a denture significantly altered the risk factors. Non-secretion of the ABH blood group antigens was an important factor among NIDDM individuals who did not wear dentures when carriage was assessed by the oral rinse.

INTRODUCTION

Oral candidiasis is most prevalent in the form of chronic atrophic candidiasis[1]. Individuals with diabetes are prone to infections; and, chronic atrophic candidiasis has been reported to be more prevalent among diabetic individuals compared with non-diabetic controls[2]. Since disease is preceded by colonization, it is important to identify factors which predispose to colonization. Few studies have examined the factors which predispose to colonization of diabetic patients by Candida albicans [2-6]; and, none of these specifically analysed patients with insulin dependent diabetes mellitus (IDDM) separately from those with non-insulin dependent diabetes mellitus (NIDDM).

Both genetic[7,8] and environmental factors[9] have been reported to affect carriage of Candida species. The genetically determined inability to secrete the glycoprotein form of the ABO blood group antigens into body fluids has been associated with susceptibility to infections by bacteria and yeasts[10,11]. Among non-diabetic individuals[7] and individuals with NIDDM[8] non-secretors of blood group O are over-represented among carriers of C. albicans compared with secretors.

Environmental factors which are variably reported to be associated with carriage of yeasts include: presence of a denture; continuous wearing of a denture; denture fit, occlusion, trauma, hygiene and age; smoking; and control of diabetes[3,12].

The aims of the present study were:

1. to compare the oral rinse technique with the palate swab method for isolating yeasts.

2. to examine in detail the species of yeasts isolated from patients with IDDM or NIDDM.

3. to assess the association between secretor status and carriage of yeasts among individuals with IDDM or NIDDM taking into account denture status.

4. to dissect the contributions of the following variables to the carriage of yeasts and, specifically, C. albicans: age; sex; type of diabetes; control of diabetes as measured by glycosylated haemoglobin A (HbA_1); random plasma glucose levels; persistent glysosuria and albuminuria; diabetic complications - retinopathy, neuropathy, nephropathy; antibiotic usage; corticosteroid treatment; smoking; alcohol consumption; presence of the denture in the mouth at night; denture fit, extension, occlusion, hygiene and age; presence of denture stomatitis; and history of superficial candidal infections.

This is the first study which is designed to compare the risk factors contributing to carriage of yeasts in individuals with IDDM or NIDDM.

MATERIALS AND METHODS

The study population, epidemiological data obtained, isolation and characterization of yeasts has been described previously[14]. Glycosylated haemoglobin A_1 (HbA_1) was measured by corning electrophoresis (normal range 4.5 - 8.0%, c.v = 4%). Results were coded, entered into a data base and the SPSSX package was used for analysis.

Statistical Analysis : the univariate analyses used were Chi square with Yates' correction and Wilcoxon rank sum test as appropriate. Frequencies of carriage of all yeasts and of C. albicans were examined separately. A P value of < 0.05 was considered significant. The stepwise linear discriminant analysis (Wilk's method) was the multivariate analysis used to identify factors best predicting carriers and non-carriers of yeasts (F - to enter > 4; $p < 0.05$).

RESULTS

Table 1 summarizes the proportion of males and females, individuals with IDDM and NIDDM, the participants' denture status, the percentage of patients with diabetic complications and those who had been treated with local and/or systemic corticosteroids and/or antibiotics within 6 months of the examination. Only 12% of the subjects complained of recurrent candida infection.

Mycological profile of the sample population

Table 2 compares the species of yeasts cultured from the five sites of the mouth and from the oral rinse for individuals with IDDM and NIDDM. In Table 3 the information presented in Table 2 is examined with reference to the presence or absence of dentures; a similar pattern was observed. T. glabrata and C. tropicalis were isolated more frequently from those with dentures. Yeasts including C. albicans were isolated more frequently from each site tested among individuals with IDDM (Table 4).

Only 29% (128/439) of the individuals examined had no yeasts in any of the five sites swabbed; 12.8% (56/439) had the same isolate cultured from all five sites. The swabbing technique compared well with the oral rinse technique in which 34% of the individuals were culture negative.

Of the 1551 isolates examined by API 20C Auxanogram Kits, 397 (25.6%) could not be reliably identified. There were 120 isolates which were identified as other than C. albicans by the kit but which produced germ tubes when incubated at 37°C in horse serum for 2 hours. Of these, 17 were sent to the Mycology Reference Laboratory at the Western General Hospital, Edinburgh for identification, and, all were found to be C. albicans (details to be reported separately).

Univariate Analysis:

Blood group and secretor status

Isolation of C. albicans or other yeasts from the palatal swab or from the oral rinse was not associated with secretor status when the results were analyzed by type of diabetes and/or denture status.

Blood group was not associated with palatal carriage of yeasts or C. albicans; however, when carriage was assessed by the oral rinse, individuals with NIDDM who wore dentures and who were of O blood group were more likely to be carriers than those of blood group A (X^2 = 7.93, $P < 0.005$) (Table 5).

Analysis of secretor status with reference to blood group revealed a significant association between non-secretion and increased frequency of carriage of yeasts among individuals with IDDM who were blood group A and who wore dentures. (9/19 A-Se carriers compared with 12/12 A-NS carriers $X^2 = 7.07$ $p = 0.008$).

Variables associated with carriage

Age: Increase in age was associated with a decreased frequency of isolation of yeasts including C. albicans from the palate of individuals with IDDM (Table 6). Results obtained from the oral rinse similarly indicated that younger individuals carried yeasts, including C. albicans, more frequently (Table 7).

Glycaemic control (HbA_1): The HbA_1 values of palatal carriers of yeasts and C. albicans were significantly higher compared with non-carriers. The contributions of type of diabetes and denture status is clearly illustrated when the sub-categories are examined (Table 8). Glycaemic control was a significant factor among individuals with IDDM but not among those with NIDDM after correcting for denture status. Subjects with IDDM who did not wear dentures were especially prone to carriage if their HbA_1 values were relatively high. The oral rinse results showed a similar overall trend but less clearly (Table 9).

Smoking: The number of cigarettes smoked per day was on the whole associated with isolation of yeasts including C. albicans from the palate (Table 10). The oral rinse results were similar except for the presence of a significant association between carriage and smoking among individuals with IDDM who did not wear dentures (Table 11).

Presence of the denture in the mouth at night: Yeasts and C. albicans were isolated more often from the palate of subjects who left their dentures in the mouth at night; however, this was observed only among individuals with NIDDM (Table 12). This pattern was not found when carriage was assessed by the oral rinse method.

Type of diabetes: Irrespective of their denture status, individuals with IDDM were more frequently carriers of yeasts compared with patients with NIDDM. Both the palate swab and the oral rinse technique results showed this trend (Table 4).

Variables not associated with frequency of isolation of yeasts: The variables listed below were not associated with frequency of carriage of C. albicans or other yeasts by either isolation technique used: sex; duration of diabetes; complications - neuropathy, nephropathy and retinopathy; alcohol consumption; contraceptive pill; systemic corticosteroid treatment; topical corticosteroid application; antibiotics; fit, occlusion or hygiene of denture; persistent albuminuria; and history of superficial candidal infections. Among individuals with NIDDM only, age of the denture and the presence of persistent glycosuria among those who did not wear dentures was significantly associated with frequency of carriage of C. albicans and yeasts in general ($P < 0.05$ in both cases). Denture status was not significantly associated with either the frequency or the density of colonization by yeasts.

Linear discriminant analysis: From the above results, it can be seen that a univariate analysis does not present a clear picture of factors contributing to carriage; associations are erratic when subgroups are analysed. In addition, univariate analysis might incorporate a dependent variable which needs prior knowledge to control for its effect.

The multivariate analysis (Tables 13 & 14) identifies factors which contribute to carriage of yeasts among denture wearing and non-denture wearing individuals with IDDM or NIDDM.

Among individuals with IDDM who wore dentures, palatal carriage of yeasts including C. albicans was associated with HbA_1 level. The variable which was the most efficient predictor of carriage when assessed by the oral rinse technique was, however, the presence of retinopathy. Univariate analysis showed HbA_1 to be associated with

the palatal carriage of C. albicans.

Individuals with IDDM who did not wear dentures were best segregated on the basis of their age into non-carriers and carriers of yeasts and in particular C. albicans. This was true when carriage was assessed by either technique. The univariate analysis showed similar associations.

Individuals with NIDDM who wore dentures were at risk of carriage of yeasts and specifically C. albicans if they smoked and, additionally, wore dentures continuously. These risk factors were identified by both sampling techniques. Comparison with univariate analysis showed that continuous wearing of dentures and smoking were significant factors only when carriage was assessed by palatal swab.

Individuals with NIDDM who did not wear dentures were more likely to be palatal carriers of C. albicans or other yeasts if they had persistent glycosuria and suffered from peripheral neuropathy. Carriage assessed by the oral rinse method was influenced by persistent glycosuria, higher random plasma glucose levels and non-secretion of blood group antigens. Univariate analysis revealed an association between persistent glucosuria and carriage among individuals with NIDDM.

DISCUSSION

The results obtained with the palate swab compared well with those obtained by the oral rinse technique. C. albicans was the species most frequently isolated from the swabs while species other than C. albicans were isolated at greater frequency from the oral rinse. This is probably due to the selective enrichment of C. albicans over other species when swabs were incubated in malt broth. Studies in which a mouth wash technique was used reported a prevalence of between 41% and 62%[2,3,5,6,13]. In this study 66% of the diabetics were carriers of yeasts by the oral rinse technique.

Yeasts were isolated from the tongue most frequently followed by the palate, floor of the mouth and the angles of the mouth. Similar results have been reported[3].

Individuals with IDDM were significantly more prone to carriage of yeasts compared to those with NIDDM. No other study has found this association[2,3,13]. This might be due to the smaller sample size; and, distinction of IDDM from NIDDM was based purely on the mode of treatment of the diabetic condition. In this study subjects were classified as IDDM or NIDDM according to clinical history of onset, requirement for insulin and progression of the disease[14].

T. glabrata and C. tropicalis were isolated more frequently from patients with either IDDM or NIDDM who wore dentures. By the API identification system, Fisher et al[5] found that the majority of isolates (89%) were C. albicans. In this study only 54% of the isolates were C. albicans; this figure includes the proportion not identified by API 20C Aux as C. albicans but were found to be so by conventional methods and compares well with other studies which found 60% of isolates to be C. albicans[3,13]. This emphasizes the need for accurate identification as patients with diabetes are more likely to carry and to have disease[14] due to species other than C. albicans which might not be sensitive to routinely prescribed oral antifungal agents[17].

The factors which were associated with the carriage of yeasts collectively were similar to those influencing carriage of C. albicans. This might be due to the majority of C. albicans in the analysis of yeast carriage.

Distinct factors influence oral carriage of yeasts and the development of disease in patients with IDDM compared with patients with NIDDM[14]. This is the first study which considers the risk factors for colonization by yeasts of individuals with IDDM as distinct from those with NIDDM taking into account various environmental factors. The two major types of diabetes appear similar; but, they differ with respect to etiology and pathogenesis as well as development of complications.

The univariate analysis showed a significantly higher proportion of blood group A non-secretors among patients with IDDM who wore dentures and were carriers of yeasts. Patients with NIDDM who were of blood group O were more frequently colonized than those of blood group A. These relationships did not appear in the multivariate analysis. Non-secretion was a marginally significant factor among patients with NIDDM who did not wear dentures. This indicates that the univariate contingency table incorporated dependent variables. The previous

study which showed NIDDM individuals prone to C. albicans carriage did not take into account the denture status of its sample population[8]. A study that did not find any association between secretor status and carriage did not examine denture wearers separately from non-denture wearers, multivariate analysis was not used and, the sample size was much too small to discern the role of secretor status which is marginal as shown above[2].

Palatal carriage of yeasts was not dependent on the secretor status. The contribution of non-secretion and carriage must therefore arise from oral sites other than the palate. This possibility is under investigation. Secretor status does, however, influence the development of disease in the palate among patients with NIDDM who are denture wearers[14].

Increase in age was associated with a decrease in the frequency of carriage probably due to the efficiency of the mucosal immune defences against yeasts with increased frequency of challenge. Age was not a significant factor in the carriage of yeasts in two studies[2,3]. Another study[6] reported an apparent increased risk of carriage of yeasts with age. The difference might be due to their smaller sample size.

Glycaemic control (HbA_1) was a significant factor associated with palatal carriage of yeasts among patients with IDDM whilst random plasma glucose level was important among those with NIDDM. This is consistent with the clinical value of HbA_1 and plasma glucose among patients with IDDM or NIDDM respectively as indicators of control of diabetes[15]. Studies that found no association between HbA_1 levels and carriage of yeasts did not differentiate between IDDM and NIDDM, and did not use multivariate analysis[2,5]. The single study in which multivariate analysis was used reported an association between HbA_1 level and increase in frequency of colonisation[6].

Random plasma glucose and HbA_1 levels were not correlated with oral disease due to yeasts[14]; but, they have a very significant effect on carriage. It would, therefore, appear that good diabetic control is a preventive measure against the eventual development of disease by reducing the probability of colonization. Although salivary levels of glucose have been reported not to correlate linearly with plasma glucose level[16], increase in plasma glucose levels also means increase in salivary levels in a non-linear relationship.

Persistent glycosuria among patients with NIDDM who did not wear dentures was isolated by both univariate and multivariate analyses as a significant factor in the carriage of yeasts. It was highly significant and indicates once more that good diabetic control reduced the possibility of colonization. Glycosuria and plasma glucose were reported to be significantly associated with yeast isolation among diabetics[13]. Other studies did not find this association[2,3,6].

Smoking was a significant risk factor for colonization among patients with NIDDM who wore dentures; however, smoking was not associated with disease development[14]. The effect of smoking is not direct since the palate is protected by the denture; but, it might have an indirect effect e.g. constriction of blood vessels and impairment of the immune system. Smoking has been reported to be associated variably with frequency of colonization when diabetics were compared

to non-diabetics[3,6].

Continuous wearing of a denture was a significant factor predisposing to colonization only among individuals with NIDDM who wore dentures. This agrees with the association between continuous presence of a denture and the development of denture stomatitis among patients with NIDDM[14] and the reported increase in the proportion of carriers among diabetics who continuously wore their dentures (70%) compared with the proportion of carriers among those who did not (44%)[3].

Retinopathy and neuropathy are specific complications of diabetes which are due to microvascular changes. Microvascular derangement might impair immune response through inadequate diffusion of tissue mediators. Both these variables were of marginal significance but indicate that systemic changes associated with diabetes predispose to colonization.

CONCLUSIONS

This study shows that:

1. Individuals with IDDM are more prone to colonization by yeasts compared to those with NIDDM. C. albicans was present in just over half the patients who were carriers of yeasts.

2. In view of the differences in factors which influence carriage and the comparative sensitivity of the two techniques, examination of the site of interest is best assessed by a swab especially as the sensitivities of the oral rinse and swab techniques were similar.

3. Incubation of a swab in malt broth selectively enhances growth of C. albicans over other species.

4. Among patients with IDDM who wear dentures, palatal carriage is influenced by HBA_1 value. Among those without dentures carriage was more frequent among younger individuals.

5. In contrast to individuals with IDDM, denture wearers with NIDDM were more frequently colonized if they smoked and, additionally, wore their dentures continuously. Those with NIDDM who did not wear dentures were at risk to carriage of yeast if they had evidence of poorly controlled glucose levels.

6. Non-secretion of ABH blood group antigens was of marginal significance in increasing the risk to carriage of yeast in the oral cavity as a whole among patients with NIDDM who did not wear dentures. Denture wearers and individuals with IDDM did not show this relationship.

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Table 1. Characteristics of the sample population

		No. (%)	Missing (%)
Sex	Males	226 (51.5)	
	Females	213 (48.5)	0
Type of diabetes	IDDM	231 (52.6)	
	NIDDM	205 (46.7)	3 (0.7)
Dentures	Full	165 (37.6)	
	Partial	50 (11.4)	
	None	221 (50.3)	3 (0.7)
	*Good fit	175 (81.4)	13 (6)
	*Good hygiene	171 (79.5)	19 (8.8)
	*Continuous wear	89 (41.4)	67 (31.6)
Presence of diabetic complications	Retinopathy	100 (23)	9 (2.1)
	Neuropathy-peripheral	73 (17)	11 (2.5)
	-autonomic	23 (5)	11 (2.5)
	Nephropathy	34 (8)	9 (2.1)
Drug consumption	Antibiotics	121 (28)	6 (1.4)
	Corticosteroids		
	- systemic	20 (4.6)	7 (1.6)
	- local	10 (2.3)	7 (1.6)
Recurrent candidiasis		54 (12)	4 (0.9)

* Percentages obtained by dividing with 215 which is the number of denture wearers.

Table 2. Species of yeast isolated from individuals with IDDM or NIDDM (% rounded to nearest whole number)

Category	Sample	None	% *C.albicans*	*T.glabrata*	*C.tropicalis*	Other
All (439)	PBS oral rinse	34	36	4	1	26
	Palate swab	47	38	4	1	9
	Tongue swab	46	43	4	1	6
	Floor of mouth swab	48	42	3	2	5
	Right angle swab	56	31	4	1	7
	Left angle swab	58	31	4	1	5
IDDM (231)	PBS oral rinse	29	45	4	0	24
	Palate swab	39	48	4	1	7
	Tongue swab	37	53	3	0	7
	Floor of mouth swab	39	53	2	2	5
	Right angle swab	54	36	3	9	7
	Left angle swab	54	37	2	0	6
NIDDM (205)	PBS oral rinse	41	25	4	2	29
	Palate swab	55	28	4	2	11
	Tongue swab	55	33	5	1	6
	Floor of mouth swab	58	29	4	2	6
	Right angle swab	59	26	6	2	7
	Left angle swab	64	24	7	2	4

Table 3. Species of yeast isolated from individuals with IDDM or NIDDM with presence or absence of dentures (% rounded to nearest whole number)

Category	Sample	% None	C.albicans	T.glabrata	C.tropicalis	Other
IDDM without dentures (150)	PBS oral rinse	33	47	2	0	29
	Palate swab	44	48	1	1	6
	Tongue swab	38	54	2	0	6
	Floor of mouth swab	41	54	1	1	3
	Right angle swab	56	38	0	0	5
	Left angle swab	60	35	0	0	5
NIDDM without dentures (70)	PBS oral rinse	40	28	2	0	31
	Palate swab	63	28	0	0	9
	Tongue swab	63	32	0	0	5
	Floor of mouth swab	70	27	0	0	3
	Right angle swab	68	27	0	0	5
	Left angle swab	75	24	0	0	2
IDDM with dentures (79)	PBS oral rinse	24	43	4	0	29
	Palate swab	33	47	9	2	9
	Tongue swab	37	49	5	2	8
	Floor of mouth swab	34	49	5	3	8
	Right angle swab	50	29	9	1	10
	Left angle swab	42	41	6	2	9
NIDDM with dentures (135)	PBS oral rinse	41	24	6	3	27
	Palate swab	51	29	6	3	12
	Tongue swab	51	34	8	2	6
	Floor of mouth swab	53	31	7	3	7
	Right angle swab	54	25	9	3	9
	Left angle swab	58	24	10	3	6

Table 4. Type of diabetes and isolation of C. albicans and other yeasts from the palatal swab or by oral rinse

Category		non-carriers	C.albicans*	Other yeasts	X^2	P
Palate swab						
ALL	IDDM	70	86	108	*13.13	0.0003
	NIDDM	101	52	82	8.49	0.004
No denture	IDDM	48	53	62	* 6.01	0.014
	NIDDM	40	18	24	5.03	0.02
Dentures	IDDM	22	31	44	* 6.23	0.013
	NIDDM	61	34	58	4.81	0.03
Oral rinse						
ALL	IDDM	57	88	139	*11.80	0.0006
	NIDDM	68	42	100	4.72	0.03
No denture	IDDM	40	57	82	* 2.84	0.092
	NIDDM	23	16	35	0.54	0.46
Dentures	IDDM	17	31	55	* 7.89	0.005
	NIDDM	45	26	65	5.05	0.025

Table 5. Carriage of yeasts and *C. albicans* assessed by the oral rinse and blood group

Category		Non-carriers	*C. albicans**	Yeasts	X^2	P
All	O	53	64	114	*0.72	0.40
	A	48	44	75	1.35	0.24
IDDM	O	27	38	60	*0.07	0.80
	A	21	35	55	0.09	0.76
NIDDM	O	26	26	54	*4.56	0.03
	A	27	9	19	7.18	0.007
ND	O	31	38	52	*0.00	1.00
	A	18	23	41	0.44	0.51
Dentures	O	22	26	62	*1.19	0.27
	A	30	21	32	6.73	0.01
IDDM non-denture wearers	O	22	26	34	*0.62	0.43
	A	11	21	32	1.49	0.22
IDDM denture wearers	O	5	12	26	*0.22	0.64
	A	10	14	22	1.24	0.27
NIDDM non-denture wearers	O	9	12	18	*1.84	0.17
	A	7	2	9	0.13	0.72
NIDDM denture wearers	O	17	14	36	*1.55	0.21
	A	20	7	10	7.93	0.005

Table 6. Age and carriage of yeasts determined by palatal swab

Category	Median (yrs) Non-carriers	*C. albicans**	Yeasts	M-W P
All	56.50	46.00	50.00	*0.00001
(n)	(170)	(137)	(189)	0.0001
IDDM	45.00	32.50	37.00	*0.002
(n)	(70)	(84)	(106)	0.02
NIDDM	60.00	55.00	56.00	*0.02
(n)	(100)	(52)	(82)	0.08
Denture wearers	61.00	55.00	56.00	*0.0005
(n)	(83)	(66)	(103)	0.002
Non-denture wearers	49.00	32.00	34.00	*0.0001
(n)	(87)	(71)	(86)	0.00001
Dentures (IDDM)	58.00	46.00	49.00	*0.03
(n)	(22)	(31)	(44)	0.033
Non-denture (IDDM)	38.50	27.00	29.00	*0.003
(n)	(48)	(53)	(62)	0.011
Dentures (NIDDM)	61.00	57.00	59.00	*0.013
(n)	(61)	(34)	(58)	0.07
Non-denture (NIDDM)	56.00	49.50	50.50	*0.03
(n)	(39)	(18)	(24)	0.09

Table 7. Age and carriage of yeasts determined by oral rinse

Category	Median (yrs) Non-carriers	*C. albicans* *	Yeasts	M-W P
All	56.50	45.00	51.00	*0.00001
(n)	(124)	(131)	(238)	0.002
IDDM	45.00	33.00	35.00	*0.05
(n)	(57)	(88)	(137)	0.12
NIDDM	60.00	55.00	57.00	*0.0007
(n)	(67)	(42)	(100)	0.123
Denture wearers	60.00	55.00	58.00	*0.003
(n)	(62)	(58)	(121)	0.07
Non-denture wearers	48.00	32.00	35.00	*0.0004
(n)	(62)	(73)	(117)	0.002
Dentures (IDDM)	56.00	53.00	53.00	*0.25
(n)	(17)	(31)	(55)	0.17
Non-denture (IDDM)	39.00	29.00	29.00	*0.006
(n)	(40)	(57)	(82)	0.013
Dentures (NIDDM)	60.00	55.55	61.00	*0.03
(n)	(45)	(26)	(65)	0.87
Non-denture (NIDDM)	57.00	52.50	54.00	*0.02
(n)	(22)	(16)	(35)	0.04

Table 8. Glycaemic control (HbA1) and carriage determined by palatal swab

Category	Median HbA1 Units Non-carriers	*C. albicans**	Yeasts	M-W P
All	9.25	10.50	10.20	*0.00001
	(160)	(121)	(167)	0.00001
IDDM	9.50	10.60	10.60	*0.0009
(n)	(63)	(76)	(93)	0.0008
NIDDM	9.10	9.80	9.60	*0.025
(n)	(97)	(45)	(73)	0.05
Denture wearers	9.30	10.50	10.20	*0.003
(n)	(75)	(57)	(89)	0.006
Non-denture wearers	9.20	10.20	10.20	0.002
(n)	(85)	(63)	(76)	0.002
Dentures (IDDM)	9.55	11.40	11.10	*0.04
(n)	(18)	(27)	(37)	0.067
Non-denture (IDDM)	9.30	10.20	10.60	*0.03
(n)	(45)	(48)	(55)	0.017
Dentures (NIDDM)	9.30	9.70	9.50	*0.13
(n)	(57)	(30)	(52)	0.15
Non-denture (NIDDM)	8.70	11.10	9.90	*0.15
(n)	(40)	(15)	(21)	0.26

Table 9. Diabetic glycaemic control (HbA1) and carriage determined by oral rinse

Category	Median HbA1 Units Non-carriers	*C. albicans**	Yeasts	M-W P
All	9.40 (117)	9.90 (119)	10.00 (214)	*0.03 0.004
IDDM (n)	9.50 (50)	10.20 (81)	10.60 (125)	*0.11 0.02
NIDDM (n)	9.30 (67)	9.50 (38)	9.55 (88)	*0.28 0.21
Denture wearers (n)	9.40 (57)	10.60 (51)	9.90 (103)	*0.03 0.11
Non-denture wearers (n)	9.35 (60)	9.75 (68)	10.00 (109)	*0.29 0.02
Dentures (IDDM) (n)	9.60 (13)	10.60 (28)	10.40 (48)	*0.66 0.68
Non-denture (IDDM) (n)	9.30 (37)	10.00 (53)	10.60 (76)	*0.149 0.016
Dentures (NIDDM) (n)	9.30 (44)	9.90 (23)	9.50 (55)	*0.06 0.28
Non-denture (NIDDM) (n)	9.40 (23)	8.90 (15)	9.60 (33)	*0.50 0.68

Table 10. Smoking and carriage of yeasts determined by palatal swab

Category	Mean cigarettes/day Non-carriers	*C. albicans**	Yeasts	M-W P
All	4.54	8.45	7.83	*0.0006
	(156)	(130)	(181)	0.0009
IDDM	6.43	18.63	8.13	*0.09
(n)	(63)	(81)	(103)	0.104
NIDDM	3.27	8.33	7.53	*0.01
(n)	(93)	(48)	(77)	0.01
Denture wearers	4.01	8.71	7.84	*0.003
(n)	(77)	(63)	(99)	0.006
Non-denture wearers	5.06	8.21	7.82	0.05
(n)	(79)	(67)	(82)	0.05
Dentures (IDDM)	7.95	10.58	9.20	*0.17
(n)	(20)	(31)	(44)	0.24
Non-denture (IDDM)	5.72	7.42	7.32	*0.421
(n)	(43)	(50)	(59)	0.43
Dentures (NIDDM)	2.89	7.13	6.87	*0.05
(n)	(57)	(31)	(54)	0.051
Non-denture (NIDDM)	4.28	10.53	9.09	*0.11
(n)	(36)	(17)	(23)	0.13

Table 11. Smoking and carriage of yeasts determined by oral rinse

Category	Mean of cigarettes/day Non-carriers	*C. albicans* *	Yeasts	M-W P
All	3.75	8.48	7.83	*0.0003
	(115)	(122)	(226)	0.0001
IDDM	4.36	8,37	8.31	*0.011
(n)	(53)	(84)	(132)	0.0063
NIDDM	3.23	8.95	7.24	*0.02
(n)	(62)	(37)	(93)	0.012
Denture wearers	4.79	9.37	7.19	*0.08
(n)	(58)	(52)	(114)	0.18
Non-denture wearers	2.68	7.81	8.48	*0.005
(n)	(57)	(70)	(112)	0.0001
Dentures (IDDM)	7.94	8.34	8.34	*0.50
(n)	(17)	((29)	(53)	0.59
Non-denture (IDDM)	2.67	8.383	8.29	*0.007
(n)	(36)	(55)	(79)	0.006
Dentures (NIDDM)	3.49	11.14	6.3	*0.04
(n)	(41)	(22)	(60)	0.33
Non-denture (NIDDM)	2.71	5.73	8.94	*0.09
(n)	(21)	(15)	(33)	0.09

Table 12. Continuous wearing of denture and isolation of yeasts from the palate

Category	Denture Present	No. (%) None	C.albicans*	Yeast	X^2	P
ALL	Yes	30 (40)	30	45 (60)	*6.05	0.014
	No	32 (65)	10	17 (35)	6.61	0.01
IDDM	Yes	10 (32)	14	21 (68)	*0.004	0.95
	No	5 (38)	5	8 (62)	0.002	0.96
NIDDM	Yes	20 (45)	16	24 (55)	*5.31	0.02
	No	27 (75)	5	9 (25)	5.97	0.015

Table 13. Variables contributing to palatal carriage assessed by Linear Stepwise Discriminant Analysis (Wilks)

	Category	%	Variables
C. albicans	IDDM with dentures (n = 41)	67	1. HBA_1
	IDDM without dentures (n = 101)	66	1. Age
	NIDDM with dentures (n = 51)	64	1. Smoking 2. Continuous denture wear
	NIDDM without dentures (n = 46)	81	1. Glycosuria
All yeasts	IDDM with dentures (n = 49)	75	1. HBA_1 2. Age 3. Plasma glucose
	NIDDM without dentures (n = 110)	64	1. Age
	NIDDM with dentures (n =72)	64	1. Smoking 2. Continuous denture wear
	NIDDM without dentures (n = 49)	80	1. Glycosuria 2. Plasma glucose 3. Peripheral neuropathy

Table 14. Variables contributing to isolation of yeasts from the oral rinse assessed by Linear Stepwise Discriminant Analysis (Wilks)

	Category	%	Variables
C. albicans	IDDM with dentures (n = 47)	62	1. Retinopathy
	IDDM without dentures (n = 97)	67	1. Age
	NIDDM with dentures (n = 51)	74	1. Smoking 2. Continuous denture wear
	NIDDM without dentures (n = 32)	72	1. Glycosuria 2. Plasma glucose 3. Secretor status
Other yeasts	IDDM with dentures (n = 71)	58	1. Retinopathy
	NIDDM without dentures (n = 122)	68	1. Age
	NIDDM with dentures (n =89)		1. Smoking 2. Continuous denture wear
	NIDDM without dentures (n = 40)	79	1. Glycosuria 2. Plasma glucose 3. Secretor status